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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ASSISTANT COMMISSIONER FOR PATENTS  
Washington, D.C. 20231

Attorney's Docket No: 05552.1337-04

Prior Application: 08/475,826

Art Unit: 1819

Examiner: B. Stanton

SIR: This is a request for filing a

☒ Continuation ☐ Divisional Application under 37 C.F.R. § 1.53(b) of pending prior application Serial No. 08/475,826 filed June 7, 1995 of Mathias Gehrmann; Gerhard Seeman; Klaus Bosslet; Jörg Czech for FUSION PROTEINS FOR PRODRUG ACTIVATION.

1. ☒ Enclosed is a complete copy of the prior application including the oath or Declaration and drawings, if any, as originally filed. I hereby verify that the attached papers are a true copy of prior application Serial No. 08/475,826 as originally filed on June 7, 1995.
2. ☐ Enclosed is a substitute specification under 37 C.F.R. § 1.125.
3. ☐ Cancel Claims \_\_\_\_\_.
4. ☐ A Preliminary Amendment is enclosed.
5. ☒ The filing fee is calculated on the basis of the claims existing in the prior application as amended at 3 and 4 above.

For	: Number Filed	: Number Extra	: Rate	: Basic Fee \$790.00
Total	:	:	:	:
Claims	: 3-20=	:	: x\$ 22.00=	: \$ -0-
Independent	:	:	:	:
Claims	: 1-3=	:	: x\$ 82.00=	: -0-
Multiple Dependent Claim(s) (if applicable)	:	:	: +\$270.00=	: -0-
Total			= :	\$790.00
Reduction by 1/2 for			:	:
filing by small entity			:	-
TOTAL FILING FEE			= :	\$790.00

LAW OFFICES  
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202-408-4000

JC491 U.S. PTO  
12/12/97

6. ☒ A check in the amount of \$1740.00 to cover the filing fee and petition for time is enclosed.
7. ☒ The Commissioner is hereby authorized to charge any fees which may be required including fees due under 37 C.F.R. § 1.16 and any other fees due under 37 C.F.R. § 1.17, or credit any overpayment during the pendency of this application to Deposit Account No. 06-0916.
8. ☒ Amend the specification by inserting before the first line, the sentence:  
  
--This is a continuation of application Serial No. 08/475,826, filed June 7, 1995, which is a divisional of Serial No. 08/404,949, filed March 15, 1995, which is a continuation of Serial No. 08/129,379, filed September 30, 1993 which is incorporated herein by reference.--
9. ☐ New formal drawings are enclosed.
10. ☒ The prior application is assigned of record to: Behringwerke Aktiengesellschaft at Reel 6738, Frames 0258-0260.
11. ☒ Priority of application Serial No. P42331528, filed on October 2, 1992 in the Federal Republic of Germany is claimed under 35 U.S.C. § 119. A certified copy is on file in the prior application.
12. ☐ A verified statement claiming small entity status  
  
☐ is enclosed or ☐ is on file in the prior application.
13. ☒ The power of attorney in the prior application is to at least one of the following: FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P., Douglas B. Henderson, Reg. No. 20,291; Ford F. Farabow, Jr., Reg. No. 20,630; Arthur S. Garrett, Reg. No. 20,338; Donald R. Dunner, Reg. No. 19,073; Brian G. Brunsvold, Reg. No. 22,593; Tipton D. Jennings, IV, Reg. No. 20,645; Jerry D. Voight, Reg. No. 23,020; Laurence R. Heffer, Reg. No. 20,827; Kenneth E. Payne, Reg. No. 23,098; Herbert H. Mintz, Reg. No. 26,691; C. Larry O'Rourke, Reg. No. 26,014; Albert J. Santorelli, Reg. No. 22,610; Michael C. Elmer, Reg. No. 25,857; Richard H. Smith, Reg. No. 20,609; Stephen L. Peterson, Reg. No. 26,325; John M. Romary, Reg. No. 26,331; Bruce C. Zotter, Reg. No. 27,680; Dennis P. O'Reilley, Reg.

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14. ☐ The power appears in the original declaration of the prior application.
15. ☐ Since the power does not appear in the original declaration, a copy of the power in the prior application is enclosed.
16. ☒ Please address all correspondence to FINNEGAN, HENDERSON, FARABOW, GARRETT and DUNNER, L.L.P., 1300 I Street, N.W., Washington, D.C. 20005-3315.
17. ☐ Recognize as associate attorney \_\_\_\_\_  
 \_\_\_\_\_  
 (name, address & Reg. No.)
18. ☐ Also enclosed is \_\_\_\_\_  
 \_\_\_\_\_

**PETITION FOR EXTENSION.** If any extension of time is necessary for the filing of this application, including any extension in the parent application, serial no. 08/475,826, filed June 7, 1995, for the purpose of maintaining copendency between the parent application and this application, and such extension has not otherwise been requested,

such an extension is hereby requested, and the Commissioner is authorized to charge necessary fees for such an extension to our Deposit Account No. 06-0916. A duplicate copy of this paper is enclosed for use in charging the deposit account.

FINNEGAN, HENDERSON, FARABOW,  
GARRETT & DUNNER, L.L.P.

By: Carol P. Einaudi  
Carol P. Einaudi  
Reg. No. 32,220

Date: December 12, 1997



GAU-PTO  
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08/989896  
PATENT  
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Attorney Docket No. 05552.1337-02

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: )  
Mathias GEHRMANN et al. )  
Serial No.: 08/475,826 ) Group Art Unit: 1819  
Filed: June 7, 1995 ) Examiner: B. Stanton  
For: FUSION PROTEINS FOR )  
PRODRUG ACTIVATION )

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

PETITION FOR EXTENSION OF TIME

Applicants hereby petition for a three month extension of time to respond to the Office Action of June 13, 1997. A fee of \$950.00 is enclosed.

If there are any other fees due in connection with the filing of this petition, please charge the fees to our Deposit Account No. 06-0916. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,  
GARRETT & DUNNER, L.L.P.

By: Carol P. Einaudi  
Carol P. Einaudi  
Reg. No. 32,220

Dated: December 12, 1997



BEHRINGWERKE AKTIENGESELLSCHAFT

92/B 024 - Ma 957

### **Fusion proteins for prodrug activation**

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The invention relates to compounds which contain an antigen binding region which is bound to at least one enzyme which is able to metabolize a compound (prodrug) which has little or no cytotoxicity to a cytotoxic compound (drug), where the antigen binding region is composed of a single polypeptide chain. It is advantageous for covalently bonded carbohydrates to be present on the polypeptide chain.

The combination of prodrug and antibody-enzyme conjugates for use as therapeutic composition has already been described in the specialist literature. This entails antibodies which are directed against a particular tissue and to which a prodrug-cleaving enzyme is bound being injected into an organism, and subsequently a prodrug compound which can be activated by the enzyme being administered. The action of the antibody-enzyme conjugate bound to the target tissue is intended to convert the prodrug compound into a compound which exerts a cytotoxic effect on the bound tissue. However, studies on antibody-enzyme conjugates have shown that these chemical conjugates have unfavorable pharmacokinetics so that there is only inadequate site-specific tumor-selective cleavage of the prodrug. Some authors have attempted to remedy this evident deficiency by additional injection of an anti-enzyme antibody which is intended to bring about rapid elimination of the antibody-enzyme conjugate from the plasma (Sharma et al.,

Brit. J. Cancer, 61, 659, 1990). Another problem of antibody-enzyme conjugates is the limited possibility of preparing large amounts reproducibly and homogeneously.

The object of the present invention was now to find fusion proteins which can be prepared on an industrial scale and are suitable, by reason of their pharmacokinetic and pharmacodynamic properties, for therapeutic uses.

It has been found in this connection that compounds which contain an antigen binding region which is composed of a single polypeptide chain have unexpected advantages for the preparation and use of fusion proteins, to which carbohydrates are advantageously attached, in prodrug activation.

The invention therefore relates to compounds which contain an antigen binding region which is bound to at least one enzyme, where the antigen binding region is composed of a single polypeptide chain, and carbohydrates are advantageously attached to the fusion protein.

An antigen binding region means for the purpose of the invention a region which contains at least two variable domains of an antibody, preferably one variable domain of a heavy antibody chain and one variable domain of a light antibody chain (sFv fragment). The antigen binding region can, however, also have a bi- or multivalent structure, i.e. two or more binding regions, as described, for example, in EP-A-0 404 097. However, a human or humanized sFv fragment is particularly preferred, especially a humanized sFv fragment.

The antigen binding region preferably binds to a tumor-associated antigen (TAA), with the following TAAs being particularly preferred:

neural cell adhesion molecule (N-CAM),  
polymorphic epithelial mucin (PEM),  
epidermal growth factor receptor (EGF-R),  
Thomsen Friedenreich antigen B (TFB),  
gastrointestinal tract carcinoma antigen (GICA),  
ganglioside GD<sub>3</sub> (GD<sub>3</sub>),  
ganglioside GD<sub>2</sub> (GD<sub>2</sub>),  
Sialyl-Le<sup>a</sup>, Sialyl-Le<sup>x</sup>,  
TAG72,  
the 24-25 kDa glycoprotein defined by MAb L6,  
CA 125 and, especially,  
carcinoembryonic antigen (CEA).

Preferred enzymes are those enzymes which are able to metabolize a compound of little or no cytotoxicity to a cytotoxic compound. Examples are  $\beta$ -lactamase, pyroglutamate aminopeptidase, galactosidase or D-aminopeptidase as described, for example, in EP-A2-0 382 411 or EP-A2-0 392 745, an oxidase such as, for example, ethanol oxidase, galactose oxidase, D-amino-acid oxidase or  $\alpha$ -glyceryl-phosphate oxidase as described, for example, in WO 91/00108, peroxidase as disclosed, for example, in EP-A2-0 361 908, a phosphatase as described, for example, in EP-A1-0 302 473, a hydroxynitrilelyase or glucosidase as disclosed, for example, in WO 91/11201, a carboxypeptidase such as, for example, carboxypeptidase G2 (WO 88/07378), an amidase such as, for example, penicillin 5-amidase (Kerr, D.E. et al. Cancer Immunol. Immunother. 1990, 31) and a protease, esterase or glycosidase such as the already mentioned galactosidase, glucosidase or a glucuronidase as described, for example, in WO 91/08770.

A  $\beta$ -glucuronidase is preferred, preferably from *Kobayasia nipponica* or *Secale cereale*, and more preferably from *E. coli* or a human  $\beta$ -glucuronidase. The substrates for the individual enzymes are also indicated in the said patents and are intended also to form part of the disclosure content of the present application. Preferred substrates of  $\beta$ glucuronidase are N-(D-glycopyranosyl)benzyloxycarbonylanthracyclines and, in particular, N-(4-hydroxy3-nitrobenzyloxycarbonyl)doxorubicin and daunorubicin  $\beta$ -D-glucuronide (J.C. Florent et al. (1992) Int. Carbohydr. Symp. Paris, A262, 297 or S. Andrianomenjanahary et al. (1992) Int. Carbohydr. Symp. Paris, A 264, 299).

The invention further relates to nucleic acids which code for the compounds according to the invention. Particularly preferred is a nucleic acid, as well as its variants and mutants, which codes for a humanized sFv fragment against CEA (carcinoembryonic antigen) linked to a human  $\beta$ -glucuronidase, preferably with the sequence indicated in Table 1 (sFv-hu $\beta$ -Gluc).

The compounds according to the invention are prepared in general by methods of genetic manipulation which are generally known to the skilled worker, it being possible for the antigen binding region to be linked to one or more enzymes either directly or via a linker, preferably a peptide linker. The peptide linker which can be used is, for example, a hinge region of an antibody or a hinge-like amino-acid sequence. In this case, the enzyme is preferably linked with the N terminus to the antigen binding region directly or via a peptide linker. The enzyme or enzymes can, however, also be linked to the antigen binding region chemically as described, for example, in WO 91/00108.

The nucleic acid coding for the amino-acid sequence of the compounds according to the invention is generally cloned in an expression vector, introduced into prokaryotic or eukaryotic host cells such as, for example, BHK, CHO, COS, HeLa, insect, tobacco plant, yeast or E.coli cells and expressed. The compound prepared in this way can subsequently be isolated and used as diagnostic aid or therapeutic agent. Another generally known method for the preparation of the compound according to the invention is the expression of the nucleic acids which code therefor in transgenic mammals with the exception of humans, preferably in a transgenic goat.

BHK cells transfected with the nucleic acids according to the invention express a fusion protein (sFv-hu $\beta$ -Gluc) which not only was specific for CEA but also had full  $\beta$ -glucuronidase activity (see Example 5).

This fusion protein was purified by anti-idiotypic affinity chromatography in accordance with the method described in EP 0 501 215 A2 (Example M). The fusion protein purified in this way gives a molecular weight of 100 kDa in the SDS PAGE under reducing conditions, while molecules of 100 and 200 kDa respectively appear under non-reducing conditions.

Gel chromatography under native conditions (TSK-3000 gel chromatography) showed one protein peak (Example 6, Fig. I) which correlates with the activity peak in the specificity enzyme activity test (EP 0 501 215 A2). The position of the peak by comparison with standard molecular weight markers indicates a molecular weight of  $\approx$  200 kDa. This finding, together with the data from the SDS PAGE, suggests that the functional enzymatically active sFv-hu $\beta$ -Gluc fusion protein is in the form of a "bivalent molecule", i.e. with 2 binding regions and 2

enzyme molecules. Experiments not described here indicate that the fusion protein may, under certain cultivation conditions, be in the form of a tetramer with 4 binding regions and 4 enzyme molecules. After the sFv-hu $\beta$ -Gluc fusion protein had been purified and undergone functional characterization in vitro, the pharmacokinetics and the tumor localization of the fusion protein were determined in nude mice provided with human gastric carcinomas. The amounts of functionally active fusion protein were determined in the organs and in the tumor at various times after appropriate workup of the organs (Example 7) and by immunological determination (triple determinant test, Example 8). The results of a representative experiment are compiled in Table 4.

Astonishingly, a tumor/plasma ratio of 5/1 is reached after only 48 hours. At later times, this ratio becomes even more favorable and reaches values > 200/1 (day 5). The reason for this favorable pharmacokinetic behavior of the sFv-hu $\beta$ -Gluc fusion protein is that fusion protein not bound to the tumor is removed from the plasma and the normal tissues by internalization mainly by receptors for mannose 6-phosphate and galactose. (Evidence for this statement is that there is an intracellular increase in the  $\beta$ -glucuronidase level, for example in the liver).

As shown in Table 5, the sFv-hu $\beta$ -Gluc contains relatively large amounts of galactose and, especially, mannose, which are mainly responsible for the binding to the particular receptors. The fusion protein/receptor complex which results and in which there is binding via the carbohydrate residues of the fusion protein is then removed from the extracellular compartment by internalization.

This rapid internalization mechanism, which is mainly mediated by galactose and mannose, is closely involved in the advantageous pharmacokinetics of the fusion protein according to the invention. These advantageous pharmacokinetics of the fusion protein to which galactose and, in particular, mannose are attached makes it possible for a hydrophilic prodrug which undergoes extracellular distribution to be administered i.v. at a relatively early time without eliciting non-specific prodrug activation. In this case an elimination step as described by Sharma et al. (Brit. J. Cancer, 61, 659, 1990) is unnecessary. Based on the data in Table 4, injection of a suitable prodrug (S. Adrianomenjanahari et al. 1992, Int. Carbohydrate Symp., Parts A264, 299) is possible even 3 days after injection of the sFv-hu $\beta$ -Gluc fusion protein without producing significant side effects (data not shown).

A similarly advantageous attachment of carbohydrates to fusion proteins can also be achieved, for example, by secretory expression of the sFv-hu $\beta$ -Gluc fusion protein in particular yeast strains such as *Saccharomyces cerevisiae* or *Hansenula polymorpha*. These organisms are capable of very effective mannosylation of fusion proteins which have appropriate N-glycosylation sites (Goochee et al., Biotechnology, 9, 1347-1354, 1991). Such fusion proteins which have undergone secretory expression in yeast cells show a high degree of mannosylation and favorable pharmacokinetics comparable to those of the sFv-hu $\beta$ -Gluc fusion protein expressed in BHK cells (data not shown). In this case, the absence of galactose is compensated by the even higher degree of mannosylation of the fusion protein (Table 6). The sFv-hu $\beta$ -Gluc fusion protein described above was constructed by genetic manipulation and expressed in yeast as described in detail in Example 9.

Instead of human  $\beta$ -glucuronidase it is, however, also possible to employ another glucuronidase with advantageous properties. For example, the E.coli  $\beta$ -glucuronidase has the particular advantage that its catalytic activity at pH 7.4 is significantly higher than that of human  $\beta$ -glucuronidase. In Example 10, an sFv-E.coli  $\beta$ -Gluc construct was prepared by methods of genetic manipulation and underwent secretory expression as functionally active mannosylated fusion protein in *Saccharomyces cerevisiae*. The pharmacokinetic data are comparable to those of the sFv-hu $\beta$ -Gluc molecule which was expressed in yeast or in BHK cells (Table 4).

The glucuronidases from the fungus *Kobayasia nipponica* and from the plants *Secale cereale* have the advantage, for example, that they are also active as monomers. In Example 11, methods of genetic manipulation were used to prepare a construct which, after expression in *Saccharomyces cerevisiae*, excretes an sFv-B. cereus  $\beta$ -lactamase II fusion protein preferentially in mannosylated form.

This fusion protein likewise has, as the fusion proteins according to the invention, on the basis of  $\beta$ -glucuronidase pharmacokinetics which are favorable for prodrug activation (Table 4).

Furthermore, the compounds according to the invention can be employed not only in combination with a prodrug but also in the framework of conventional chemotherapy in which cytostatics which are metabolized as glucuronides and thus inactivated can be converted back into their toxic form by the administered compounds.

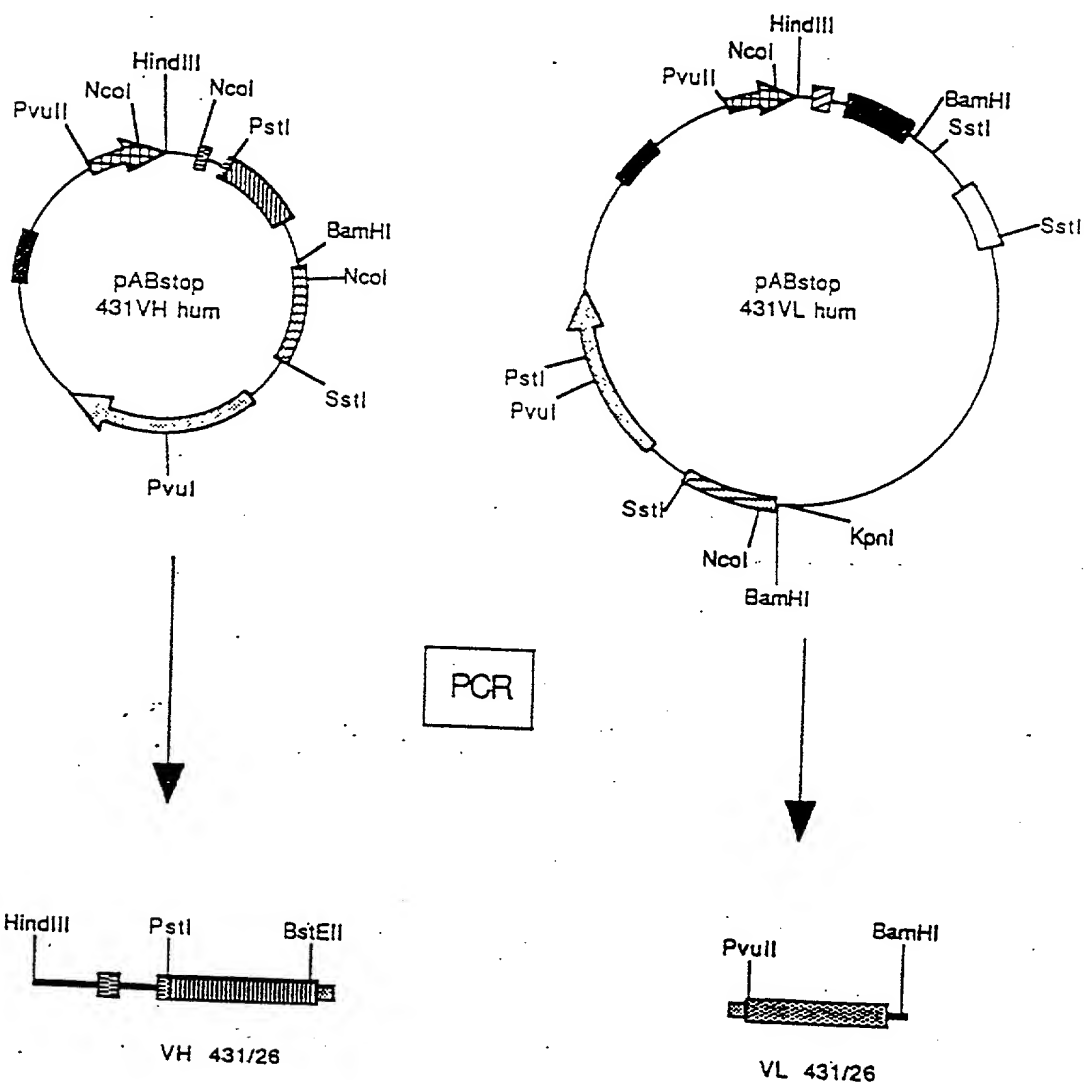
The following examples now describe the synthesis by genetic manipulation of sFv- $\beta$ -Gluc fusion proteins, and the demonstration of the ability to function.

The starting material comprised the plasmids pABstop 431/26 hum V<sub>H</sub> and pABstop 431/26 hum V<sub>H</sub>L. These plasmids contain the humanized version of the V<sub>H</sub> gene and V<sub>L</sub> gene of anti-CEA Mab BW 431/26 (Güssow and Seemann, 1991, Meth. Enzymology, 203, 99-121). Further starting material comprised the plasmid pABstop 431/26 V<sub>H</sub>-hu $\beta$ -Gluc 1H (EP-A2-0 501 215) which contains a V<sub>H</sub> exon, including the V<sub>H</sub>-intrinsic signal sequence, followed by a CH1 exon, by the hinge exon of a human IgG3 C gene and the complete cDNA of human  $\beta$ -glucuronidase.

**Example 1:**

**Amplification of the  $V_H$  and  $V_L$  genes of MAb hum 431/26**

The oligonucleotides pAB-Back and linker-anti (Tab. 2) are used to amplify the  $V_H$  gene including the signal sequence intrinsic to the  $V_H$  gene from pABstop 431 $V_H$  hum ( $V_H$  431/26) (Güssow and Seemann, 1991, Meth. Enzymology, 203, 99-121). The oligonucleotides linker-sense and  $V_L$ (Mut)-For (Tab. 3) are used to amplify the  $V_L$  gene from pABstop 431 $V_L$  hum ( $V_L$  431/26).



Example 2:

Joining of the  $V_H$  431/26 and  $V_L$  431/26 gene fragments

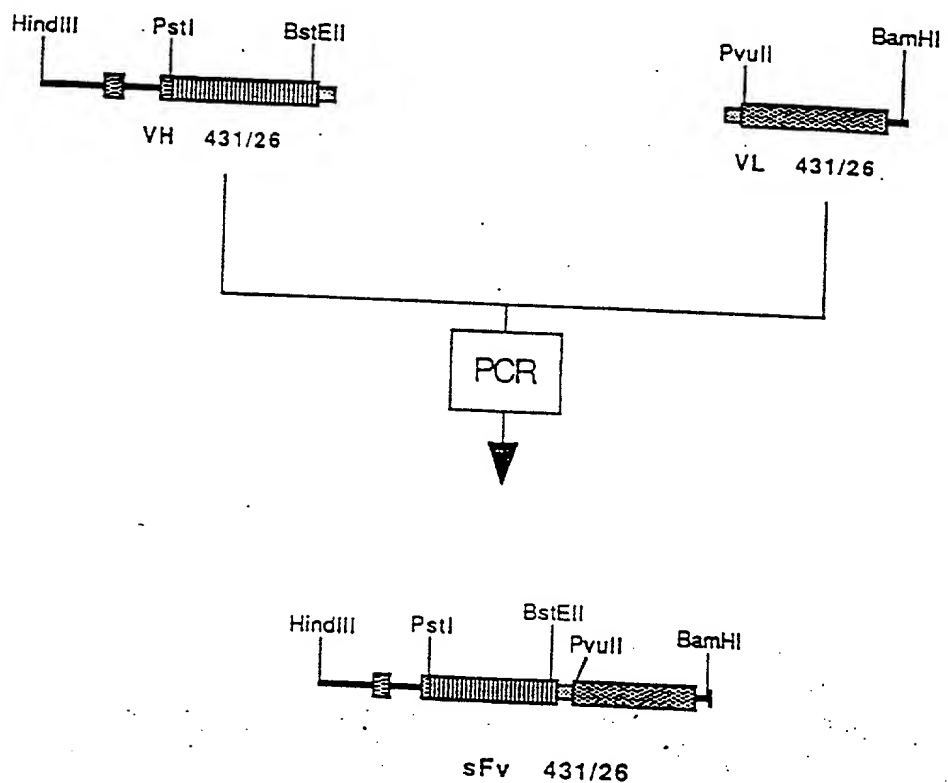
The oligonucleotides linker-anti and linker-sense are partially complementary with one another and encode a polypeptide linker which is intended to link the  $V_H$  domain and  $V_L$  domain to give an sFv fragment. In order to fuse the amplified  $V_H$  fragments with the  $V_L$  fragments, they are purified and employed in a 10-cycle reaction as follows:

$H_2O$ :	37.5 $\mu l$
dNTPs (2.5 mM):	5.0 $\mu l$
PCR buffer (10x):	5.0 $\mu l$
Taq polymerase (Perkin-Elmer Corp., Emmeryville, CA)	
(2.5 U/ $\mu l$ ):	0.5 $\mu l$
0.5 $\mu g/\mu l$ DNA of the $V_L$ frag.:	1.0 $\mu l$
0.5 $\mu g/\mu l$ DNA of the $V_H$ frag.:	1.0 $\mu l$

PCR buffer (10x): 100 mM tris, pH 8.3, 500 mM KCl, 15 mM  $MgCl_2$ , 0.1% (w/v) gelatin.

The surface of the reaction mixture is sealed with paraffin, and subsequently the 10-cycle reaction is carried out in a PCR apparatus programmed for 94°C, 1 min; 55°C, 1 min; 72°C, 2 min. 2.5 pmol of the flanking primer pAB-Back and  $V_{L(Mut)}$ -For are added, and a further 20 cycles are carried out. The resulting PCR fragment is composed of the  $V_H$  gene which is linked to the  $V_L$  gene via a linker. The signal sequence intrinsic to the  $V_H$  gene is also present in front of the  $V_H$  gene.

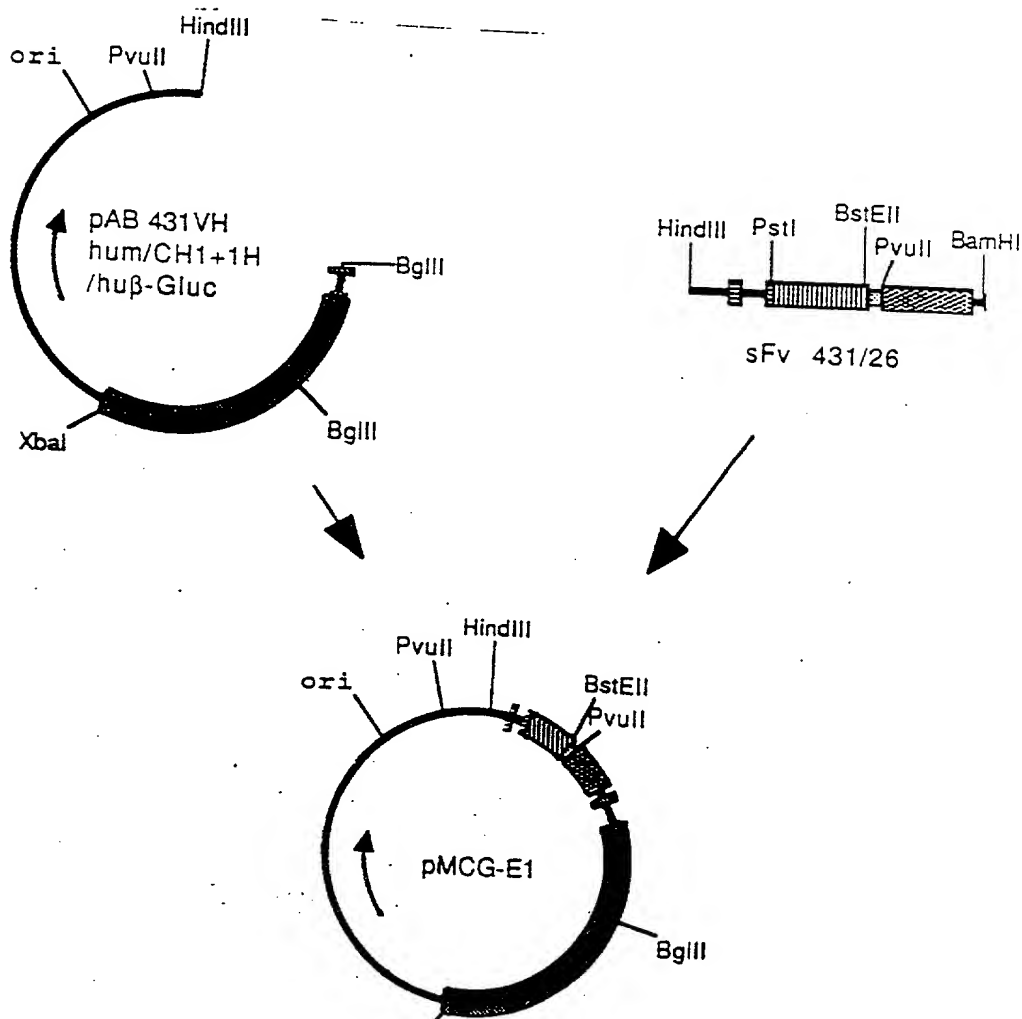
The oligonucleotide  $V_{L(Mut)}$ -For also results in the last nucleotide base of the  $V_L$  gene, a C, being replaced by a G. This PCR fragment codes for a humanized single-chain Fv (sFv 431/26).



**Example 3:**

**Cloning of the sFv 431/26 fragment into the expression vector which contains the hu $\beta$ -glucuronidase gene.**

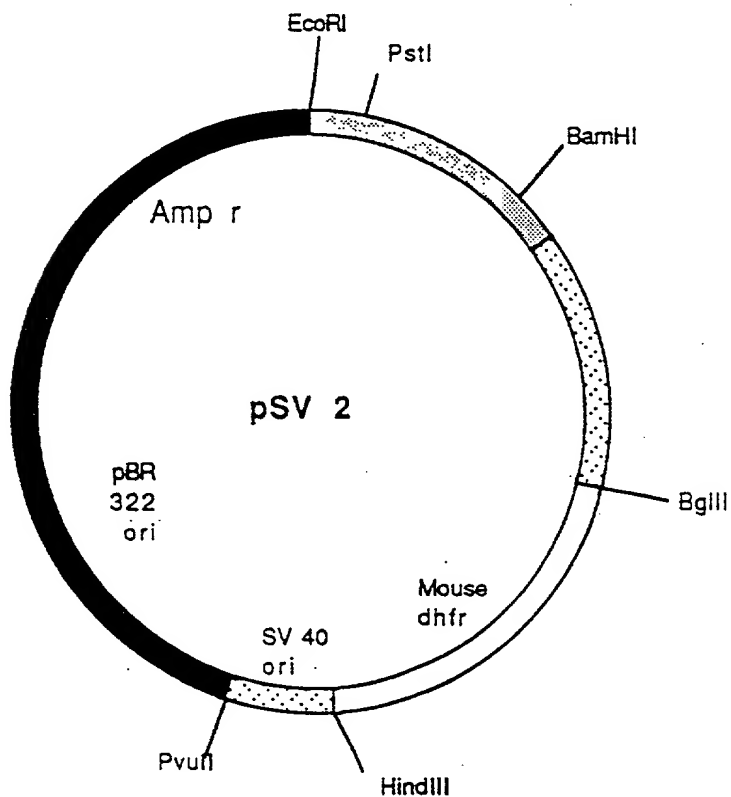
The sFv fragment from (2) is cut with HindIII and BamHI and ligated into the vector pAB 431V<sub>H</sub> hum/CH1 + 1h/ $\beta$ -Glc which has been completely cleaved with HindIII and partially cleaved with BglII. The vector pABstop 431/26V<sub>H</sub>hu $\beta$ -Gluc1H contains a V<sub>H</sub> exon, including the V<sub>H</sub>-intrinsic signal sequence, followed by a CH1 exon, by the hinge exon of a human IgG3 C gene and by the complete cDNA of human  $\beta$ -glucuronidase. The plasmid clone pMCG-E1 which contains the humanized sFv 431/26, a hinge exon and the gene for human  $\beta$ -glucuronidase is isolated (pMCG-E1).

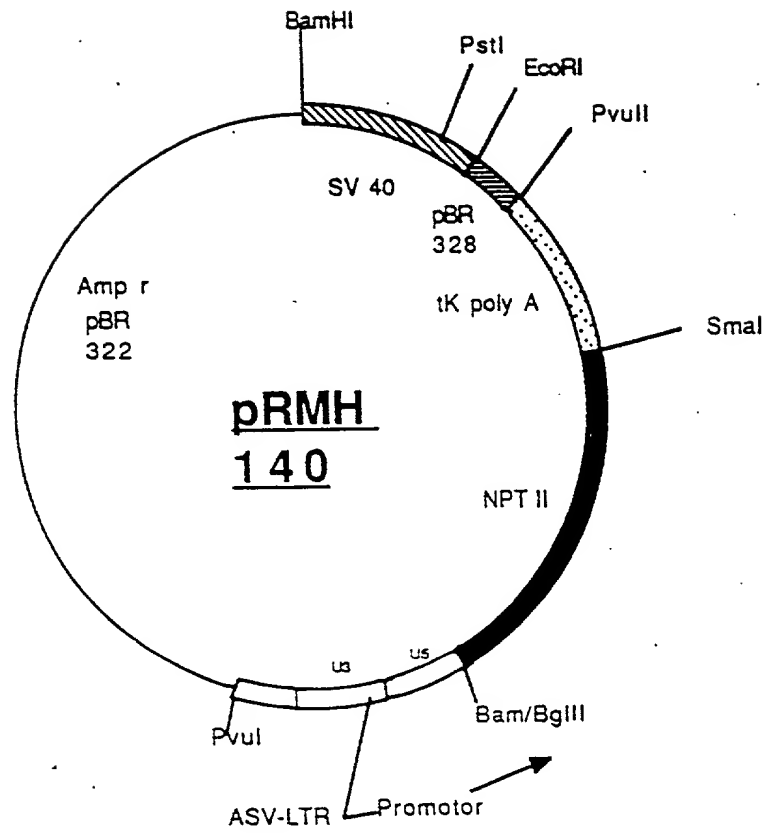


**Example 4:**

**Expression of the sFv-hu $\beta$ -Gluc fusion protein in BHK cells.**

The clone pMCG-E1 is transfected with the plasmid pRMH 140 which harbors a neomycin-resistance gene and with the plasmid pSV2 which harbors a methotrexateresistance gene into BHK cells. The BHK cells subsequently express a fusion protein which has both the antigen-binding properties of MAb BW 431/26hum and the enzymatic activity of human  $\beta$ -glucuronidase.





**Example 5:**

**Demonstration of the antigen-binding properties and of the enzymatic activity of the sFv-hu $\beta$ -Gluc fusion protein.**

The ability of the sFv-hu $\beta$ -Gluc fusion protein to bind specifically to the CEA epitope defined by 431/26 and simultaneously to exert the enzymatic activity of human  $\beta$ -glucuronidase was shown in a specificity enzyme activity test (EP-A2-0 501 215). The test determines the liberation of 4-methylumbelliferone from 4-methylumbelliferyl  $\beta$ -glucuronide by the  $\beta$ -glucuronidase portion of the fusion protein after the fusion protein has been bound via the sFv portion to an antigen. The measured fluorescence values are reported as relative fluorescence units (FU). The test shows a significant liberation of methyl-umbelliferone by the fusion protein in the plates coated with CEA. By contrast, the fusion protein does not liberate any methylumbelliferone in control plates coated with PEM (polymorphic epithelial mucin).

Example 6:

**TSK 3000 gel chromatography**

200 ng of the sFv-hu $\beta$ -Gluc fusion protein which had been purified by anti-idiotypic affinity chromatography in 25  $\mu$ l were chromatographed on a TSK gel G 3000 SW XL column (TOSO HAAS Order No. 3.5Wx N3211, 7.8 mm x 300 mm) in a suitable mobile phase (PBS, pH 7.2, containing 5 g/l maltose and 4.2 g/l arginine) at a flow rate of 0.5 ml/ min. The Merck Hitachi HPLC system (L-4000 UV detector, L-6210 intelligent pump, D-2500 Chromato-integrator) was operated under  $\approx$  20 bar, the optical density of the eluate was determined at 280 nm, and an LKB 2111 Multisac fraction collector was used to collect 0.5 ml fractions which were subsequently analysed in a specificity enzyme activity test (SEAT) (EP 0 501 215 A2, Example J). The result of this experiment is shown in Fig. 1. It is clearly evident that the position of the peak detectable by measurement of the optical density at 280 nm coincides with the peak which determines the specificity and enzyme activity (SEAT) of the eluate. Based on the positions of the molecular weights of standard proteins which are indicated by arrows, it can be concluded that the functionally active sFv-hu $\beta$ -Gluc fusion protein has an approximate molecular weight of  $\approx$  200 kDa under native conditions.

**Example 7:**

**Workup of organs/tumors for determination of the fusion protein**

The following sequential steps were carried out:

- nude mice (CD1) which have a subcutaneous tumor and have been treated with fusion protein or antibody-enzyme conjugate undergo retroorbital exsanguination and are then sacrificed
- the blood is immediately placed in an Eppendorf tube which already contains 10  $\mu$ l of Liquemin 25000 (from Hoffman-LaRoche AG)
- centrifugation is then carried out in a centrifuge (Megafuge 1.0, from Heraeus) at 2500 rpm for 10 min
- the plasma is then obtained and frozen until tested
- the organs or the tumor are removed and weighed
- they are then completely homogenized with 2 ml of 1% BSA in PBS, pH 7.2
- the tumor homogenates are adjusted to pH 4.2 with 0.1 N HCl (the sample must not be overtitrated because  $\beta$ -glucuronidase is inactivated at pH < 3.8)
- all the homogenates are centrifuged at 16000 g for 30 min
- the clear supernatant is removed
- the tumor supernatants are neutralized with 0.1 N NaOH
- the supernatants and the plasma can now be quantified in immunological tests.

**Example 8:**

**Triple determinant test**

The tests are carried out as follows:

- 75  $\mu$ l of a mouse anti-hu $\beta$ -Gluc antibody (MAb 2118/157 Behringwerke) diluted to 2  $\mu$ g/ml in PBS, pH 7.2, are placed in each well of a microtiter plate (polystyrene U-shape, type B, from Nunc, Order No. 4-60445)
- the microtiter plates are covered and incubated at R.T. overnight
- the microtiter plates are subsequently washed 3x with 250  $\mu$ l of 0.05 M tris-citrate buffer, pH 7.4, per well
- these microtiter plates coated in this way are incubated with 250  $\mu$ l of blocking solution (1% casein in PBS, pH 7.2) in each well at R.T. for 30' (blocking of non-specific binding sites) (coated microtiter plates which are not required are dried at R.T. for 24 hours and then sealed together with drying cartridges in coated aluminum bags for long-term storage)
- during the blocking, in an untreated 96-well U-shaped microtiter plate (polystyrene, from Renner, Order No. 12058), 10 samples + 2 positive controls + 1 negative control are diluted 1:2 in 1% casein in PBS, pH 7.2, in 8 stages (starting from 150  $\mu$ l of sample, 75  $\mu$ l of sample are pipetted into 75  $\mu$ l of casein solution etc.)
- the blocking solution is aspirated out of the microtiter plate coated with anti-hu $\beta$ -Gluc antibodies, and 50  $\mu$ l of the diluted samples are transferred per well from the dilution plate to the test plate and incubated at R.T. for 30 min

- during the sample incubation, the ABC-AP reagent (from Vectastain, Order No. AK-5000) is made up: thoroughly mix 2 drops of reagent A (Avidin DH) in 10 ml of 1% casein in PBS, pH 7.2, add 2 drops of reagent B (biotinylated alkaline phosphatase) add mix thoroughly. (The ABC-AP solution must be made up at least 30' before use.)
- the test plate is washed 3 times with ELISA washing buffer (Behringwerke, Order No. OSEW 96)
- 50  $\mu$ l of biotin-labeled detecting antibody mixture (1 + 1 mixture of mouse anti 431/26 antibody (MAb 2064/353, Behringwerke) and mouse anti-CEA antibody (MAb 250/183, Behringwerke) in a concentration of 5  $\mu$ g/ml diluted in 1% casein in PBS, pH 7.2, final concentration of each antibody of 2.5  $\mu$ g/ml) are placed in each well
- the test plate is washed 3 times with ELISA washing buffer
- 50  $\mu$ l of the prepared ABC-AP solution are placed in each well and incubated at R.T. for 30 min
- during the ABC-AP incubation, the substrate is made up (fresh substrate for each test: 1 mM 4-methylumbelliferyl phosphate, Order No. M-8883, from Sigma, in 0.5 M tris + 0.01%  $MgCl_2$ , pH 9.6)
- the test plate is washed 7 times with ELISA washing buffer
- 50  $\mu$ l of substrate are loaded into each well, and the test plate is covered and incubated at 37°C for 2 h
- 150  $\mu$ l of stop solution (0.2 M glycine + 0.2% SDS, pH 11.7) are subsequently added to each well
- the fluorometric evaluation is carried out in a Fluoroscan II (ICN Biomedicals, Cat.No. 78-611-00) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm

- the unknown concentration of fusion protein in the sample is determined on the basis of the fluorescence values for the positive control included in the identical experiment (dilution series with purified sFv-hu $\beta$ -Gluc mixed with CEA 5  $\mu$ g/ml as calibration plot).

Example 9:

**Expression of the sFv-hu $\beta$ -Gluc fusion protein in yeast.**

The single-chain Fv (sFv) from Example 2 is amplified with the oligos 2577 and 2561 (Table 7) and cloned into the vector pUC19 which has been digested with XbaI/HindIII (Fig. 2).

The human  $\beta$ -glucuronidase gene is amplified with the oligos 2562 and 2540 (Table 8) from the plasmid pAB 431/26 V<sub>H</sub>hum/CH1 + 1H/ $\beta$ -Gluc (Example 3) and ligated into the plasmid sFv 431/26 in pUC19 (Fig. 2) cut with BglIII/HindIII (Fig. 3).

A KpnI/NcoI fragment is amplified with the oligos 2587 and 2627 (Table 9) from the sFv 431/26 and cloned into the yeast expression vector pIXY digested with KpnI/NcoI (Fig. 4).

The BstEII/HindIII fragment from the plasmid sFv 431/26 hu $\beta$ -Gluc in pUC19 (Fig. 3) is ligated into the vector pIXY 120 which harbors the V<sub>H</sub> gene, the linker and a part of the V<sub>L</sub> gene (V<sub>H</sub>/link/V<sub>K</sub> part. in pIXY 120) and has been digested with BstEII/partially with HindIII (Fig. 5).

The resulting plasmid sFv 431/26 hu $\beta$ -Gluc in pIXY 120 is transformed into *Saccharomyces cerevisiae* and the fusion protein is expressed.

Example 10:

**Expression of the sFv-E.coli- $\beta$ -glucuronidase fusion protein in yeast.**

The E.coli glucuronidase gene is amplified from pRAJ 275 (Jefferson et al. Proc. Natl. Acad. Sci, USA, 83: 8447-8451, 1986) with the oligos 2638 and 2639 (Table 10) and ligated into sFv 431/26 in pUC19 (Example 9, Fig. 2) cut with BglIII/HindIII (Fig. 6).

A BstEII/HindIII fragment from sFv 431/26 E.coli  $\beta$ -Gluc in pUC19 is cloned into the vector  $V_H$ /link/ $V_K$  part in pIXY 120 (Example 9, Fig. 4) which has been partially digested with BstEII/HindIII (Fig. 7).

The plasmid sFv 431/26 E.coli  $\beta$ -Gluc in pIXY 120 is transformed into *Saccharomyces cerevisiae* and the fusion protein is expressed.

Example 11:

**Expression of the sFv- $\beta$ -lactamase fusion protein in yeast.**

The single-chain Fv (sFv) from Example 2 is amplified with the oligos 2587 and 2669 (Table 11) and ligated into the pUC19 vector digested with KpnI/HindIII (Fig. 8).

The  $\beta$ -lactamase II gene (Hussain et al., J. Bacteriol. 164: 223-229, 1985) is amplified with the oligos 2673 and 2674 (Table 11) from the complete DNA of *Bacillus cereus* and ligated into the pUC19 vector digested with EcoRI/HindIII (Fig. 9). A BclI/HindIII fragment of the  $\beta$ -lactamase gene is ligated into sFv 431/26 in pUC19 which has been cut with BglII/HindIII (Fig. 10).

The KpnI/HindIII sFv- $\beta$ -lactamase fragment is ligated into pIXY 120 which has been digested with KpnI/partially with HindIII (Fig. 11). The plasmid is transformed into *Saccharomyces cerevisiae*, and a fusion protein which has both the antigen-binding properties of MAb 431/26 and the enzymatic activity of *Bacillus cereus*  $\beta$ -lactamase is expressed.

Table 1:

CCAAGCTTAT GAATATGCAA ATCCTGCTCA TGAATATGCA AATCCTCTGA	50
ATCTACATGG TAAATATAGG TTTGTCTATA CCACAAACAG AAAAACATGA	100
GATCACAGTT CTCTCTACAG TTAGTGAGCA CACAGGACCT CACC ATG GGA TGG Met Gly Trp	153
AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGTAAGGGGC Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr	199
-10	
TCACAGTAGC AGGCTTGAGG TCTGGACATA TATATGGGTG ACAATGACAT	249
CCACTTTGCC TTTCTCTCCA CA GGT GTC CAC TCC CAG GTC CAA CTG CAG Gly Val His Ser Gln Val Gln Leu Gln	298
1	
GAG AGC GGT CCA GGT CTT GTG AGA CCT AGC CAG ACC CTG AGC CTG Glu Ser Gly Pro Gly Leu Val Arg Pro Ser Gln Thr Leu Ser Leu	343
10 20	
ACC TGC ACC GTG TCT GGC TTC ACC ATC AGC AGT GGT TAT AGC TGG Thr Cys Thr Val Ser Gly Phe Thr Ile Ser Ser Gly Tyr Ser Trp	388
30	
CAC TGG GTG AGA CAG CCA CCT GGA CGA GGT CTT GAG TGG ATT GGA His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp Ile Gly	433
40 50	
TAC ATA CAG TAC AGT GGT ATC ACT AAC TAC AAC CCC TCT CTC AAA Tyr Ile Gln Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu Lys	478
60	
AGT AGA GTG ACA ATG CTG GTA GAC ACC AGC AAG AAC CAG TTC AGC Ser Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn Gln Phe Ser	523
70 80	
CTG AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC GCG GTC TAT TAT Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr	568
90	
TGT GCA AGA GAA GAC TAT GAT TAC CAC TGG TAC TTC GAT GTC TGG Cys Ala Arg Glu Asp Tyr Asp Tyr His Trp Tyr Phe Asp Val Trp	613
100 110	
GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA GGA GGC GGT GGA TCG Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser	658
120	
GGC GGT GGT GGG TCG GGT GGC GGC GGA TCT GAC ATC CAG CTG ACC Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr	703
130 140	
CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT GAC AGA GTG ACC Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr	748
150	
ATC ACC TGT AGT ACC AGC TCG AGT GTA AGT TAC ATG CAC TGG TAC Ile Thr Cys Ser Thr Ser Ser Ser Val Ser Tyr Met His Trp Tyr	793
160 170	
CAG CAG AAG CCA GGT AAG GCT CCA AAG CTG CTG ATC TAC AGC ACA Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ser Thr	838
180	

Table 1 (Continuation):

TCC AAC CTG GCT TCT GGT GTG CCA AGC AGA TTC AGC GGT AGC GGT	883
Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly	
190 200	
AGC GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC CTC CAG CCA GAG	928
Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu	
210	
GAC ATC GCC ACC TAC TAC TGC CAT CAG TGG AGT AGT TAT CCC ACG	973
Asp Ile Ala Thr Tyr Tyr Cys His Gln Trp Ser Ser Tyr Pro Thr	
220 230	
TTC GGC CAA GGG ACC AAG CTG GAG ATC AAA GGTGAGTAGA ATTTAAACTT	1023
Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys	
240	
TGCTTCCTCA GTTGGATCTG AGTAACTCCC AATCTTCTCT CTGCA GAG CTC AAA	1077
Glu Leu Lys	
ACC CCA CTT GGT GAC ACA ACT CAC ACA TGC CCA CGG TGC CCA	1119
Thr Pro Leu Gly Asp Thr Thr His Thr Cys Pro Arg Cys Pro	
250	
GGTAAGCCAG CCCAGGACTC GCCCTCCAGC TCAAGGCGGG ACAAGAGCCC	1169
TAGAGTGGCC TGAGTCCAGG GACAGGCCCC AGCAGGGTGC TGACGCATCC	1219
ACCTCCATCC CAGATCCCCG TAACTCCCAA TCTTCTCTCT GCA GCG GCG GCG	1271
Ala Ala Ala	
260	
GCG GTG CAG GGC GGG ATG CTG TAC CCC CAG GAG AGC CCG TCG CGG	1316
Ala Val Gln Gly Gly Met Leu Tyr Pro Gln Glu Ser Pro Ser Arg	
270	
GAG TGC AAG GAG CTG GAC GGC CTC TGG AGC TTC CGC GCC GAC TTC	1361
Glu Cys Lys Glu Leu Asp Gly Leu Trp Ser Phe Arg Ala Asp Phe	
280 290	
TCT GAC AAC CGA CGC CGG GGC TTC GAG GAG CAG TGG TAC CGG CGG	1406
Ser Asp Asn Arg Arg Arg Gly Phe Glu Glu Gln Trp Tyr Arg Arg	
300	
CCG CTG TGG GAG TCA GGC CCC ACC GTG GAC ATG CCA GTT CCC TCC	1451
Pro Leu Trp Glu Ser Gly Pro Thr Val Asp Met Pro Val Pro Ser	
310 320	
AGC TTC AAT GAC ATC AGC CAG GAC TGG CGT CTG CGG CAT TTT GTC	1496
Ser Phe Asn Asp Ile Ser Gln Asp Trp Arg Leu Arg His Phe Val	
330	
GGC TGG GTG TGG TAC GAA CGG GAG GTG ATC CTG CCG GAG CGA TGG	1541
Gly Trp Val Trp Tyr Glu Arg Glu Val Ile Leu Pro Glu Arg Trp	
340 350	
ACC CAG GAC CTG CGC ACA AGA GTG GTG CTG AGG ATT GGC AGT GCC	1586
Thr Gln Asp Leu Arg Thr Arg Val Val Leu Arg Ile Gly Ser Ala	
360	
CAT TCC TAT GCC ATC GTG TGG GTG AAT GGG GTC GAC ACG CTA GAG	1631
His Ser Tyr Ala Ile Val Trp Val Asn Gly Val Asp Thr Leu Glu	
370 380	
CAT GAG GGG GGC TAC CTC CCC TTC GAG GCC GAC ATC AGC AAC CTG	1676
His Glu Gly Gly Tyr Leu Pro Phe Glu Ala Asp Ile Ser Asn Leu	
390	
GTC CAG GTG GGG CCC CTG CCC TCC CGG CTC CGA ATC ACT ATC GCC	1721
Val Gln Val Gly Pro Leu Pro Ser Arg Leu Arg Ile Thr Ile Ala	
400 410	

Table 1 (Continuation):

ATC	AAC	AAC	ACA	CTC	ACC	CCC	ACC	ACC	CTG	CCA	CCA	GGG	ACC	ATC	1766
Ile	Asn	Asn	Thr	Leu	Thr	Pro	Thr	Thr	Leu	Pro	Pro	Gly	Thr	Ile	
									420						
CAA	TAC	CTG	ACT	GAC	ACC	TCC	AAG	TAT	CCC	AAG	GGT	TAC	TTT	GTC	1811
Gln	Tyr	Leu	Thr	Asp	Thr	Ser	Lys	Tyr	Pro	Lys	Gly	Tyr	Phe	Val	
				430										440	
CAG	AAC	ACA	TAT	TTT	GAC	TTT	TTC	AAC	TAC	GCT	GGA	CTG	CAG	CGG	1856
Gln	Asn	Thr	Tyr	Phe	Asp	Phe	Phe	Asn	Tyr	Ala	Gly	Leu	Gln	Arg	
									450						
TCT	GTA	CTT	CTG	TAC	ACG	ACA	CCC	ACC	ACC	TAC	ATC	GAT	GAC	ATC	1901
Ser	Val	Leu	Leu	Tyr	Thr	Thr	Pro	Thr	Thr	Tyr	Ile	Asp	Asp	Ile	
				460										470	
ACC	GTC	ACC	ACC	AGC	GTG	GAG	CAA	GAC	AGT	GGG	CTG	GTG	AAT	TAC	1946
Thr	Val	Thr	Thr	Ser	Val	Glu	Gln	Asp	Ser	Gly	Leu	Val	Asn	Tyr	
									480						
CAG	ATC	TCT	GTC	AAG	GGC	AGT	AAC	CTG	TTC	AAG	TTG	GAA	GTG	CGT	1991
Gln	Ile	Ser	Val	Lys	Gly	Ser	Asn	Leu	Phe	Lys	Leu	Glu	Val	Arg	
				490										500	
CTT	TTG	GAT	GCA	GAA	AAC	AAA	GTC	GTG	GCG	AAT	GGG	ACT	GGG	ACC	2036
Leu	Leu	Asp	Ala	Glu	Asn	Lys	Val	Val	Ala	Asn	Gly	Thr	Gly	Thr	
									510						
CAG	GGC	CAA	CTT	AAG	GTG	CCA	GGT	GTC	AGC	CTC	TGG	TGG	CCG	TAC	2081
Gln	Gly	Gln	Leu	Lys	Val	Pro	Gly	Val	Ser	Leu	Trp	Trp	Pro	Tyr	
				520										530	
CTG	ATG	CAC	GAA	CGC	CCT	GCC	TAT	CTG	TAT	TCA	TTG	GAG	GTG	CAG	2126
Leu	Met	His	Glu	Arg	Pro	Ala	Tyr	Leu	Tyr	Ser	Leu	Glu	Val	Gln	
									540						
CTG	ACT	GCA	CAG	ACG	TCA	CTG	GGG	CCT	GTG	TCT	GAC	TTC	TAC	ACA	2171
Leu	Thr	Ala	Gln	Thr	Ser	Leu	Gly	Pro	Val	Ser	Asp	Phe	Tyr	Thr	
				550										560	
CTC	CCT	GTG	GGG	ATC	CGC	ACT	GTG	GCT	GTC	ACC	AAG	AGC	CAG	TTC	2216
Leu	Pro	Val	Gly	Ile	Arg	Thr	Val	Ala	Val	Thr	Lys	Ser	Gln	Phe	
									570						
CTC	ATC	AAT	GGG	AAA	CCT	TTC	TAT	TTC	CAC	GGT	GTC	AAC	AAG	CAT	2261
Leu	Ile	Asn	Gly	Lys	Pro	Phe	Tyr	Phe	His	Gly	Val	Asn	Lys	His	
				580										590	
GAG	GAT	GCG	GAC	ATC	CGA	GGG	AAG	GGC	TTC	GAC	TGG	CCG	CTG	CTG	2306
Glu	Asp	Ala	Asp	Ile	Arg	Gly	Lys	Gly	Phe	Asp	Trp	Pro	Leu	Leu	
									600						
GTG	AAG	GAC	TTC	AAC	CTG	CTT	CGC	TGG	CTT	GGT	GCC	AAC	GCT	TTC	2351
Val	Lys	Asp	Phe	Asn	Leu	Leu	Arg	Trp	Leu	Gly	Ala	Asn	Ala	Phe	
				610										620	
CGT	ACC	AGC	CAC	TAC	CCC	TAT	GCA	GAG	GAA	GTG	ATG	CAG	ATG	TGT	2396
Arg	Thr	Ser	His	Tyr	Pro	Tyr	Ala	Glu	Glu	Val	Met	Gln	Met	Cys	
									630						
GAC	CGC	TAT	GGG	ATT	GTG	GTC	ATC	GAT	GAG	TGT	CCC	GGC	GTG	GGC	2441
Asp	Arg	Tyr	Gly	Ile	Val	Val	Ile	Asp	Glu	Cys	Pro	Gly	Val	Gly	
				640										650	
CTG	GCG	CTG	CCG	CAG	TTC	TTC	AAC	AAC	GTT	TCT	CTG	CAT	CAC	CAC	2486
Leu	Ala	Leu	Pro	Gln	Phe	Phe	Asn	Asn	Val	Ser	Leu	His	His	His	
									660						
ATG	CAG	GTG	ATG	GAA	GAA	GTG	GTG	CGT	AGG	GAC	AAG	AAC	CAC	CCC	2531
Met	Gln	Val	Met	Glu	Glu	Val	Val	Arg	Arg	Asp	Lys	Asn	His	Pro	
				670										680	

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GCG	ATC	GTC	ATG	TGG	TCT	GTG	GCC	AAC	GAG	CCT	GCG	TCC	CAC	CTA	2576
Ala	Val	Val	Met	Trp	Ser	Val	Ala	Asn	Glu	Pro	Ala	Ser	His	Leu	
									690						
GAA	TCT	GCT	GGC	TAC	TAC	TTG	AAG	ATG	GTG	ATC	GCT	CAC	ACC	AAA	2621
Glu	Ser	Ala	Gly	Tyr	Tyr	Leu	Lys	Met	Val	Ile	Ala	His	Thr	Lys	
				700										710	
TCC	TTG	GAC	CCC	TCC	CGG	CCT	GTG	ACC	TTT	GTG	AGC	AAC	TCT	AAC	2666
Ser	Leu	Asp	Pro	Ser	Arg	Pro	Val	Thr	Phe	Val	Ser	Asn	Ser	Asn	
									720						
TAT	GCA	GCA	GAC	AAG	GGG	GCT	CCG	TAT	GTG	GAT	GTG	ATC	TGT	TTG	2711
Tyr	Ala	Ala	Asp	Lys	Gly	Ala	Pro	Tyr	Val	Asp	Val	Ile	Cys	Leu	
				730										740	
AAC	AGC	TAC	TAC	TCT	TGG	TAT	CAC	GAC	TAC	GGG	CAC	CTG	GAG	TTG	2756
Asn	Ser	Tyr	Tyr	Ser	Trp	Tyr	His	Asp	Tyr	Gly	His	Leu	Glu	Leu	
									750						
ATT	CAG	CTG	CAG	CTG	GCC	ACC	CAG	TTT	GAG	AAC	TGG	TAT	AAG	AAG	2801
Ile	Gln	Leu	Gln	Leu	Ala	Thr	Gln	Phe	Glu	Asn	Trp	Tyr	Lys	Lys	
				760										770	
TAT	CAG	AAG	CCC	ATT	ATT	CAG	AGC	GAG	TAT	GGA	GCA	GAA	ACG	ATT	2846
Tyr	Gln	Lys	Pro	Ile	Ile	Gln	Ser	Glu	Tyr	Gly	Ala	Glu	Thr	Ile	
									780						
GCA	GGG	TTT	CAC	CAG	GAT	CCA	CCT	CTG	ATG	TTC	ACT	GAA	GAG	TAC	2891
Ala	Gly	Phe	His	Gln	Asp	Pro	Pro	Leu	Met	Phe	Thr	Glu	Glu	Tyr	
				790										800	
CAG	AAA	AGT	CTG	CTA	GAG	CAG	TAC	CAT	CTG	GGT	CTG	GAT	CAA	AAA	2936
Gln	Lys	Ser	Leu	Leu	Glu	Gln	Tyr	His	Leu	Gly	Leu	Asp	Gln	Lys	
									810						
CGC	AGA	AAA	TAT	GTG	GTT	GGA	GAG	CTC	ATT	TGG	AAT	TTT	GCC	GAT	2981
Arg	Arg	Lys	Tyr	Val	Val	Gly	Glu	Leu	Ile	Trp	Asn	Phe	Ala	Asp	
				820										830	
TTC	ATG	ACT	GAA	CAG	TCA	CCG	ACG	AGA	GTG	CTG	GGG	ATT	AAA	AAG	3026
Phe	Met	Thr	Glu	Gln	Ser	Pro	Thr	Arg	Val	Leu	Gly	Asn	Lys	Lys	
									840						
GGG	ATC	TTC	ACT	CGG	CAG	AGA	CAA	CCA	AAA	AGT	GCA	GCG	TTC	CTT	3071
Gly	Ile	Phe	Thr	Arg	Gln	Arg	Gln	Pro	Lys	Ser	Ala	Ala	Phe	Leu	
				850										860	
TTG	CGA	GAG	AGA	TAC	TGG	AAG	ATT	GCC	AAT	GAA	ACC	AGG	TAT	CCC	3116
Leu	Arg	Glu	Arg	Tyr	Trp	Lys	Ile	Ala	Asn	Glu	Thr	Arg	Tyr	Pro	
									870						
CAC	TCA	GTA	GCC	AAG	TCA	CAA	TGT	TTG	GAA	AAC	AGC	CCG	TTT	ACT	3161
His	Ser	Val	Ala	Lys	Ser	Gln	Cys	Leu	Glu	Asn	Ser	Pro	Phe	Thr	
				880										890	
TGA	GCAAGACTGA	TACCACCTGC	GTGTCCCTTC												

Table 2:

pAB-Back:

<sup>5'</sup> ACC AGA AGC TTA TGA ATA TGC AAA TC' <sup>3'</sup>

Linker-Anti:

5' GCC ACC CGA CCC ACC ACC GCC CGA TCC ACC GCC TCC TGA  
GGA GAC GGT GAC CGT GGT C 3'

Table 3:

Linker-Sense:

5' GGT GGA TCG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCT  
GAC ATC CAG CTG ACC CAG AGC 3'

VL(Mut)-For:

5' TGC AGG ATC CAA CTG AGG AAG CAA AGT TTA AAT TCT ACT  
3' CAC CTT TGA TC

Table 4

Pharmacokinetics of sFv-hu $\beta$ Gluc fusion protein in CD1 nu/nu mice carrying MzStol							
ng of sFv-hu $\beta$ Gluc per gram of tissue or ml of plasma measured in the triple determinant test							
Tissue type	Mouse 1 0.05 h	Mouse 2 3 h	Mouse 3 24 h	Mouse 4 48 h	Mouse 5a 120 h	Mouse 5b 120 h	
Tumor	24.8	4	7.7	2.1	2.2	6.2	
Spleen	15.4	4.1	<0.1	<0.1	<0.1	<0.1	
Liver	40.9	10.1	0.8	0.8	0.3	<0.1	
Intestine	5.2	4.4	1.1	1.2	0.6	<0.1	
Kidney	44.4	7	<0.1	<0.1	<0.1	<0.1	
Lung	154.8	17.3	<0.1	<0.1	<0.1	<0.1	
Heart	148.3	8.2	<0.1	<0.1	<0.1	<0.1	
Plasma	630.9	95	2.7	0.4	<0.1	<0.1	

i.v. injection of 0.8  $\mu$ g of purified fusion protein per mouse

Table 5

Analysis of the monosaccharide components in the carbohydrate content of the sFv-hu $\beta$ -Gluc fusion protein from BHK cells

The purified sFv-hu $\beta$ -Gluc fusion protein was investigated for its carbohydrate content. This revealed after hydrolysis the following individual components in the stated molar ratio (mol of carbohydrate/mol of sFv-hu $\beta$ -Gluc).

	Fucose	Galactosamine	N-Acetyl glucosamine	Galactose	Glucose	Mannose	N-Acetyl-neuraminic acid
sFv-hu $\beta$ -Gluc	4	2	30	8	1	43	4

The molar ratios of mannose, glucosamine and galactose allow conclusions to be drawn about the presence of the high-mannose type and/or hybrid type structures (besides complex type structures). Therefore mannose, galactose, acetylneuraminic acid and possibly N-acetylglucosamine occur terminally, and mannose may also be present as mannose 6-phosphate.

#### Methods:

**Neuraminic acid was determined** by the method of Hermentin and Seidat (1991) GBF Monographs Volume 15, pp. 185-188 (after hydrolysis for 30 min in the presence of 0.1 N sulfuric acid at 80 °C and subsequent neutralization with 0.4 N sodium hydroxide solution) by high-pH anion exchange chromatography with pulsed amperometric detection (HPAE-PAD).

The **monosaccharide components** were determined (after hydrolysis for 4 h in the presence of 2 N trifluoroacetic acid at 100 °C and evaporation to dryness in a SpeedVac) likewise by HPAE-PAD in a motivation of the method described by Hardy et al. (1988) Analytical Biochemistry 170, pp. 54-62.

Table 6

Analysis of the monosaccharide components in the carbohydrate content of the sFv-huβGluc fusion protein from *Saccharomyces cerevisiae*.

	Glucosamine	Glucose	Mannose	
sFv-huβGluc (mol/mol)	6	12	150	mol/mol

Table 7:

Oligos for sFv 431/26 cloning in pUC 19

sFv for (2561)

5' TTT TTA AGC TTA GAT CTC CAC CTT GGT C 3'

5 sFv back (2577)

5' AAA AAT CTA GAA TGC AGG TCC AAC TGC AGG  
AGA G 3'

Table 8:

Oligos for hum.β-Gluc cloning in sFv pUC 19

10 Hum.β-Gluc. back oligo (2562)

5' AAA AAA GTG ATC AAA GCG TCT GGC GGG CCA CAG  
GGC GGG ATC CTG TAC 3'

Hum.β-Gluc for oligo (2540)

5' TTT TAA GCT TCA AGT AAA CGG GCT GTT 3'



Table 10:

Oligos for E.coli  $\beta$ -Gluc cloning in sFv pUC 19

E. coli  $\beta$ -Gluc. for (2639)

5'    TTT TAA GCT TCC ATG GCG GCC GCT CAT TGT TTG  
5       CCT CCC TGC TG            3'

E. coli  $\beta$ -Gluc. back (2638)

5'    AAA AAG ATC TCC GCG TCT GGC GGG CCA CAG TTA  
      CGT GTA GAA ACC CCA            3'

Table 11:

Oligos for sFv/ $\beta$ -lactamase cloning in pIXY120

PCR oligo VHpIXY back (2587)

5'    TTT TGG TAC CTT TGG ATA AAA GAC AGG TCC AAC TGC AGG  
5       AGA G       3'

PCR oligo VKpIXY/ $\beta$ -lactamase for (2669)

5'    AAA AAG CTT AGA TCT CCA GCT TGG TCC C    3'

PCR oligo link/ $\beta$ -lactamase back (2673)

5'    AAA GAA TTC TGA TCA AAT CCT CGA GCT CAG GTT CAC  
10       AAA AGG TAG AGA AAA CAG T    3' linker

PCR oligo  $\beta$ -lactamase for (2674)

5'    TTT AAG CTT ATT TTA ATA AAT CCA ATG T    3'

Patent Claims for USA:

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1. A compound containing an antigen binding region which is bound to at least one prodrug-activating enzyme, where the antigen binding region is composed of a single polypeptide chain.
2. A compound as claimed in claim 1, wherein the compound carries covalently bonded carbohydrates.
3. A compound as claimed in claim 1, wherein the antigen binding region contains a variable domain of a heavy antibody chain and a variable domain of a light antibody chain (sFv fragment).
4. A compound as claimed in claim 1, wherein the antigen binding region binds to a tumor-associated antigen (TAA).
5. A compound as claimed in claim 3, wherein the TAA is an N-CAM, PEM, EGF-R, Sialyl-Le<sup>a</sup>, Sialyl-Le<sup>x</sup>, TFB, GICA, GD<sub>3</sub>, GD<sub>2</sub>, TAG72, CA125, the 24-25 kDa glycoprotein defined by MAb L6, or CEA, preferably a CEA.
6. A compound as claimed in claim 1, wherein the enzyme is a lactamase, preferably a *Bacillus cereus* II  $\beta$ -lactamase, pyroglutamate aminopeptidase, D-aminopeptidase, oxidase, peroxidase, phosphatase, hydroxynitrile lyase, protease, esterase, carboxypeptidase, preferably a carboxypeptidase G2 from *Pseudomonas* or glycosidase.

7. A compound as claimed in claim 6, wherein the enzyme is a  $\beta$ -glucuronidase, preferably a *E.coli*, *Kobayasia nipponica*, *Secale cereale* or human  $\beta$ -glucuronidase.
8. A compound as claimed in claim 1, wherein the antigen binding region is linked to the enzyme via a peptide linker.
9. A compound as claimed in claim 1, wherein the glycosylation takes place either by means of chemical methods or by a selection of suitable expression systems.
10. A compound as claimed in claim 1, which undergoes secretory expression in *Saccharomyces cerevisiae* or, more advantageously, in *Hansenula polymorpha*.
11. A compound as claimed in claim 1, which is expressed in *E. coli* and is subsequently chemically glycosylated, preferably galactosylated and/or mannosylated.
12. A compound as claimed in claim 1, wherein the sFv- $\beta$ -lactamase fusion protein, which has undergone periplasmic expression in *E. coli*, is chemically glycosylated, preferably galactosylated and/or mannosylated.
13. A compound as claimed in claim 1, wherein the sFv- $\beta$ -lactamase fusion protein undergoes secretory expression in *Saccharomyces cerevisiae* or *Hansenula polymorpha*.
14. A nucleic acid coding for a compound as claimed in claim 1.

15. A nucleic acid as claimed in claim 14, coding for a humanized sFv fragment against CEA and a human  $\beta$ -glucuronidase.

16. A nucleic acid as claimed in claim 14 with the sequence

CCAAGCTTAT GAATATGCAA ATCCTGCTCA TGAATATGCA AATCCTCTGA	50
ATCTACATGG TAAATATAGG TTTGTCTATA CCACAAACAG AAAAACATGA	100
GATCACAGTT CTCTCTACAG TTAGTGAGCA CACAGGACCT CACC ATG GGA TGG	153
Met Gly Trp	
AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGTAAGGGGC	199
Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr	
-10	
TCACAGTAGC AGGCTTGAGG TCTGGACATA TATATGGGTG ACAATGACAT	249
CCACTTTGCC TTTCTCTCCA CA GGT GTC CAC TCC CAG GTC CAA CTG CAG	298
Gly Val His Ser Gln Val Gln Leu Gln	
1	
GAG AGC GGT CCA GGT CTT GTG AGA CCT AGC CAG ACC CTG AGC CTG	343
Glu Ser Gly Pro Gly Leu Val Arg Pro Ser Gln Thr Leu Ser Leu	
10 20	
ACC TGC ACC GTG TCT GGC TTC ACC ATC AGC AGT GGT TAT AGC TGG	388
Thr Cys Thr Val Ser Gly Phe Thr Ile Ser Ser Gly Tyr Ser Trp	
30	
CAC TGG GTG AGA CAG CCA CCT GGA CGA GGT CTT GAG TGG ATT GGA	433
His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp Ile Gly	
40 50	
TAC ATA CAG TAC AGT GGT ATC ACT AAC TAC AAC CCC TCT CTC AAA	478
Tyr Ile Gln Tyr Ser Gly Ile Thr Asn Tyr Asn Pro Ser Leu Lys	
60	
AGT AGA GTG ACA ATG CTG GTA GAC ACC AGC AAG AAC CAG TTC AGC	523
Ser Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn Gln Phe Ser	
70 80	
CTG AGA CTC AGC AGC GTG ACA GCC GCC GAC ACC GCG GTC TAT TAT	568
Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr	
90	
TGT GCA AGA GAA GAC TAT GAT TAC CAC TGG TAC TTC GAT GTC TGG	613
Cys Ala Arg Glu Asp Tyr Asp Tyr His Trp Tyr Phe Asp Val Trp	
100 110	
GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA GGA GGC GGT GGA TCG	658
Gly Gln Gly Thr Thr Val Thr Val Ser Ser Gly Gly Gly Gly Ser	
120	
GGC GGT GGT GGG TCG GGT GGC GGC GGA TCT GAC ATC CAG CTG ACC	703
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Leu Thr	
130 140	
CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT GAC AGA GTG ACC	748
Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr	
150	

ATC ACC TGT AGT ACC AGC TCG AGT GTA AGT TAC ATG CAC TGG TAC	793
Ile Thr Cys Ser Thr Ser Ser Ser Val Ser Tyr Met His Trp Tyr	
160	170
CAG CAG AAG CCA GGT AAG GCT CCA AAG CTG CTG ATC TAC AGC ACA	838
Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Ser Thr	
180	
TCC AAC CTG GCT TCT GGT GTG CCA AGC AGA TTC AGC GGT AGC GGT	883
Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly	
190	200
AGC GGT ACC GAC TTC ACC TTC ACC ATC AGC AGC CTC CAG CCA GAG	928
Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu	
210	
GAC ATC GCC ACC TAC TAC TGC CAT CAG TGG AGT AGT TAT CCC ACG	973
Asp Ile Ala Thr Tyr Tyr Cys His Gln Trp Ser Ser Tyr Pro Thr	
220	230
TTC GGC CAA GGG ACC AAG CTG GAG ATC AAA GGTGAGTAGA ATTTAAACTT	1023
Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys	
240	
TGCTTCCTCA GTTGGATCTG AGTAACTCCC AATCTTCTCT CTGCA GAG CTC AAA	1077
Glu Leu Lys	
ACC CCA CTT GGT GAC ACA ACT CAC ACA TGC CCA CGG TGC CCA	1119
Thr Pro Leu Gly Asp Thr Thr His Thr Cys Pro Arg Cys Pro	
250	
GGTAAGCCAG CCCAGGACTC GCCCTCCAGC TCAAGGCGGG ACAAGAGCCC	1169
TAGAGTGGCC TGAGTCCAGG GACAGGCCCC AGCAGGGTGC TGACGCATCC	1219
ACCTCCATCC CAGATCCCCG TAACTCCCAA TCTTCTCTCT GCA GCG GCG GCG	1271
Ala Ala Ala	
260	
GCG GTG CAG GGC GGG ATG CTG TAC CCC CAG GAG AGC CCG TCG CGG	1316
Ala Val Gln Gly Gly Met Leu Tyr Pro Gln Glu Ser Pro Ser Arg	
270	
GAG TGC AAG GAG CTG GAC GGC CTC TGG AGC TTC CGC GCC GAC TTC	1361
Glu Cys Lys Glu Leu Asp Gly Leu Trp Ser Phe Arg Ala Asp Phe	
280	290
TCT GAC AAC CGA CGC CGG GGC TTC GAG GAG CAG TGG TAC CGG CGG	1406
Ser Asp Asn Arg Arg Arg Gly Phe Glu Glu Gln Trp Tyr Arg Arg	
300	
CCG CTG TGG GAG TCA GGC CCC ACC GTG GAC ATG CCA GTT CCC TCC	1451
Pro Leu Trp Glu Ser Gly Pro Thr Val Asp Met Pro Val Pro Ser	
310	320
AGC TTC AAT GAC ATC AGC CAG GAC TGG CGT CTG CGG CAT TTT GTC	1496
Ser Phe Asn Asp Ile Ser Gln Asp Trp Arg Leu Arg His Phe Val	
330	
GGC TGG GTG TGG TAC GAA CGG GAG GTG ATC CTG CCG GAG CGA TGG	1541
Gly Trp Val Trp Tyr Glu Arg Glu Val Ile Leu Pro Glu Arg Trp	
340	350
ACC CAG GAC CTG CGC ACA AGA GTG GTG CTG AGG ATT GGC AGT GCC	1586
Thr Gln Asp Leu Arg Thr Arg Val Val Leu Arg Ile Gly Ser Ala	
360	
CAT TCC TAT GCC ATC GTG TGG GTG AAT GGG GTC GAC ACG CTA GAG	1631
His Ser Tyr Ala Ile Val Trp Val Asn Gly Val Asp Thr Leu Glu	
370	380

CAT	GAG	GGG	GGC	TAC	CTC	CCC	TTC	GAG	GCC	GAC	ATC	AGC	AAC	CTG	1676
His	Glu	Gly	Gly	Tyr	Leu	Pro	Phe	Glu	Ala	Asp	Ile	Ser	Asn	Leu	
									390						
GTC	CAG	GTG	GGG	CCC	CTG	CCC	TCC	CGG	CTC	CGA	ATC	ACT	ATC	GCC	1721
Val	Gln	Val	Gly	Pro	Leu	Pro	Ser	Arg	Leu	Arg	Ile	Thr	Ile	Ala	
				400										410	
ATC	AAC	AAC	ACA	CTC	ACC	CCC	ACC	ACC	CTG	CCA	CCA	GGG	ACC	ATC	1766
Ile	Asn	Asn	Thr	Leu	Thr	Pro	Thr	Thr	Leu	Pro	Pro	Gly	Thr	Ile	
									420						
CAA	TAC	CTG	ACT	GAC	ACC	TCC	AAG	TAT	CCC	AAG	GGT	TAC	TTT	GTC	1811
Gln	Tyr	Leu	Thr	Asp	Thr	Ser	Lys	Tyr	Pro	Lys	Gly	Tyr	Phe	Val	
				430										440	
CAG	AAC	ACA	TAT	TTT	GAC	TTT	TTC	AAC	TAC	GCT	GGA	CTG	CAG	CGG	1856
Gln	Asn	Thr	Tyr	Phe	Asp	Phe	Phe	Asn	Tyr	Ala	Gly	Leu	Gln	Arg	
									450						
TCT	GTA	CTT	CTG	TAC	ACG	ACA	CCC	ACC	ACC	TAC	ATC	GAT	GAC	ATC	1901
Ser	Val	Leu	Leu	Tyr	Thr	Thr	Pro	Thr	Thr	Tyr	Ile	Asp	Asp	Ile	
				460										470	
ACC	GTC	ACC	ACC	AGC	GTG	GAG	CAA	GAC	AGT	GGG	CTG	GTG	AAT	TAC	1946
Thr	Val	Thr	Thr	Ser	Val	Glu	Gln	Asp	Ser	Gly	Leu	Val	Asn	Tyr	
									480						
CAG	ATC	TCT	GTC	AAG	GGC	AGT	AAC	CTG	TTC	AAG	TTG	GAA	GTG	CGT	1991
Gln	Ile	Ser	Val	Lys	Gly	Ser	Asn	Leu	Phe	Lys	Leu	Glu	Val	Arg	
				490										500	
CTT	TTG	GAT	GCA	GAA	AAC	AAA	GTC	GTG	GCG	AAT	GGG	ACT	GGG	ACC	2036
Leu	Leu	Asp	Ala	Glu	Asn	Lys	Val	Val	Ala	Asn	Gly	Thr	Gly	Thr	
									510						
CAG	GGC	CAA	CTT	AAG	GTG	CCA	GGT	GTC	AGC	CTC	TGG	TGG	CCG	TAC	2081
Gln	Gly	Gln	Leu	Lys	Val	Pro	Gly	Val	Ser	Leu	Trp	Trp	Pro	Tyr	
				520										530	
CTG	ATG	CAC	GAA	CGC	CCT	GCC	TAT	CTG	TAT	TCA	TTG	GAG	GTG	CAG	2126
Leu	Met	His	Glu	Arg	Pro	Ala	Tyr	Leu	Tyr	Ser	Leu	Glu	Val	Gln	
									540						
CTG	ACT	GCA	CAG	ACG	TCA	CTG	GGG	CCT	GTG	TCT	GAC	TTC	TAC	ACA	2171
Leu	Thr	Ala	Gln	Thr	Ser	Leu	Gly	Pro	Val	Ser	Asp	Phe	Tyr	Thr	
				550										560	
CTC	CCT	GTG	GGG	ATC	CGC	ACT	GTG	GCT	GTC	ACC	AAG	AGC	CAG	TTC	2216
Leu	Pro	Val	Gly	Ile	Arg	Thr	Val	Ala	Val	Thr	Lys	Ser	Gln	Phe	
									570						
CTC	ATC	AAT	GGG	AAA	CCT	TTC	TAT	TTC	CAC	GGT	GTC	AAC	AAG	CAT	2261
Leu	Ile	Asn	Gly	Lys	Pro	Phe	Tyr	Phe	His	Gly	Val	Asn	Lys	His	
				580										590	
GAG	GAT	GCG	GAC	ATC	CGA	GGG	AAG	GGC	TTC	GAC	TGG	CCG	CTG	CTG	2306
Glu	Asp	Ala	Asp	Ile	Arg	Gly	Lys	Gly	Phe	Asp	Trp	Pro	Leu	Leu	
									600						
GTG	AAG	GAC	TTC	AAC	CTG	CTT	CGC	TGG	CTT	GGT	GCC	AAC	GCT	TTC	2351
Val	Lys	Asp	Phe	Asn	Leu	Leu	Arg	Trp	Leu	Gly	Ala	Asn	Ala	Phe	
				610										620	
CGT	ACC	AGC	CAC	TAC	CCC	TAT	GCA	GAG	GAA	GTG	ATG	CAG	ATG	TGT	2396
Arg	Thr	Ser	His	Tyr	Pro	Tyr	Ala	Glu	Glu	Val	Met	Gln	Met	Cys	
									630						
GAC	CGC	TAT	GGG	ATT	GTG	GTC	ATC	GAT	GAG	TGT	CCC	GGC	GTG	GGC	2441
Asp	Arg	Tyr	Gly	Ile	Val	Val	Ile	Asp	Glu	Cys	Pro	Gly	Val	Gly	
				640										650	

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CTG GCG CTG CCG CAG TTC TTC AAC AAC GTT TCT CTG CAT CAC CAC	2486
Leu Ala Leu Pro Gln Phe Phe Asn Asn Val Ser Leu His His His	
660	
ATG CAG GTG ATG GAA GAA GTG GTG CGT AGG GAC AAG AAC CAC CCC	2531
Met Gln Val Met Glu Glu Val Val Arg Arg Asp Lys Asn His Pro	
670	680
GCG GTC GTG ATG TGG TCT GTG GCC AAC GAG CCT GCG TCC CAC CTA	2576
Ala Val Val Met Trp Ser Val Ala Asn Glu Pro Ala Ser His Leu	
690	
GAA TCT GCT GGC TAC TAC TTG AAG ATG GTG ATC GCT CAC ACC AAA	2621
Glu Ser Ala Gly Tyr Tyr Leu Lys Met Val Ile Ala His Thr Lys	
700	710
TCC TTG GAC CCC TCC CGG CCT GTG ACC TTT GTG AGC AAC TCT AAC	2666
Ser Leu Asp Pro Ser Arg Pro Val Thr Phe Val Ser Asn Ser Asn	
720	
TAT GCA GCA GAC AAG GGG GCT CCG TAT GTG GAT GTG ATC TGT TTG	2711
Tyr Ala Ala Asp Lys Gly Ala Pro Tyr Val Asp Val Ile Cys Leu	
730	740
AAC AGC TAC TAC TCT TGG TAT CAC GAC TAC GGG CAC CTG GAG TTG	2756
Asn Ser Tyr Tyr Ser Trp Tyr His Asp Tyr Gly His Leu Glu Leu	
750	
ATT CAG CTG CAG CTG GCC ACC CAG TTT GAG AAC TGG TAT AAG AAG	2801
Ile Gln Leu Gln Leu Ala Thr Gln Phe Glu Asn Trp Tyr Lys Lys	
760	770
TAT CAG AAG CCC ATT ATT CAG AGC GAG TAT GGA GCA GAA ACG ATT	2846
Tyr Gln Lys Pro Ile Ile Gln Ser Glu Tyr Gly Ala Glu Thr Ile	
780	
GCA GGG TTT CAC CAG GAT CCA CCT CTG ATG TTC ACT GAA GAG TAC	2891
Ala Gly Phe His Gln Asp Pro Pro Leu Met Phe Thr Glu Glu Tyr	
790	800
CAG AAA AGT CTG CTA GAG CAG TAC CAT CTG GGT CTG GAT CAA AAA	2936
Gln Lys Ser Leu Leu Glu Gln Tyr His Leu Gly Leu Asp Gln Lys	
810	
CGC AGA AAA TAT GTG GTT GGA GAG CTC ATT TGG AAT TTT GCC GAT	2981
Arg Arg Lys Tyr Val Val Gly Glu Leu Ile Trp Asn Phe Ala Asp	
820	830
TTC ATG ACT GAA CAG TCA CCG ACG AGA GTG CTG GGG ATT AAA AAG	3026
Phe Met Thr Glu Gln Ser Pro Thr Arg Val Leu Gly Asn Lys Lys	
840	
GGG ATC TTC ACT CGG CAG AGA CAA CCA AAA AGT GCA GCG TTC CTT	3071
Gly Ile Phe Thr Arg Gln Arg Gln Pro Lys Ser Ala Ala Phe Leu	
850	860
TTG CGA GAG AGA TAC TGG AAG ATT GCC AAT GAA ACC AGG TAT CCC	3116
Leu Arg Glu Arg Tyr Trp Lys Ile Ala Asn Glu Thr Arg Tyr Pro	
870	
CAC TCA GTA GCC AAG TCA CAA TGT TTG GAA AAC AGC CCG TTT ACT	3161
His Ser Val Ala Lys Ser Gln Cys Leu Glu Asn Ser Pro Phe Thr	
880	890
TGA GCAAGACTGA TACCACCTGC GTGTCCCTTC CTCCCCGAGT CAGGGCGACT	3214
...	
TCCACAGCAG CAGAACAAGT GCCTCCTGGA CTGTTACGG CAGACCAGAA	3264
CGTTTCTGGC CTGGGTTTTG TGGTCATCTA TTCTAGCAGG GAACACTAAA	3314

17. A vector containing a nucleic acid as claimed in claim 14.
18. A host cell containing a nucleic acid as claimed in claim 14 or a vector as claimed in claim 17.
19. A host cell as claimed in claim 18, which is a BHK, CHO, COS, HeLa, insect, tobacco plant, yeast or E.coli cell.
20. A transgenic mammal with the exception of a human, containing a DNA as claimed in claim 14 or a vector as claimed in claim 17.
21. A process for preparing a compound as claimed in claim 1, which comprises
  - a) introducing a nucleic acid as claimed in claim 14 or a vector as claimed in claim 17 into a host cell,
  - b) cultivating the host cell, and
  - c) isolating the compound.
22. A process for preparing a compound as claimed in claim 1, which comprises
  - a) cultivating a host cell as claimed in claim 18, and
  - b) isolating the compound.

23. The use of the compound as claimed in claim 1 for the preparation of a pharmaceutical or of a diagnostic aid.
24. The use of the compound as claimed in claim 1 for the preparation of a pharmaceutical for the treatment of cancer.
25. A pharmaceutical containing a compound as claimed in claim 1.
26. A diagnostic aid containing a compound as claimed in claim 1.

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## Fusion proteins for prodrug activation

The invention relates to compounds which contain an antigen binding region which is bound to at least one enzyme which is able to metabolize a compound (prodrug) which has little or no cytotoxicity to a cytotoxic compound (drug), where the antigen binding region is composed of a single polypeptide chain. It is advantageous for covalently bonded carbohydrates to be present on the polypeptide chain.

TSK 3000 gel chromatography

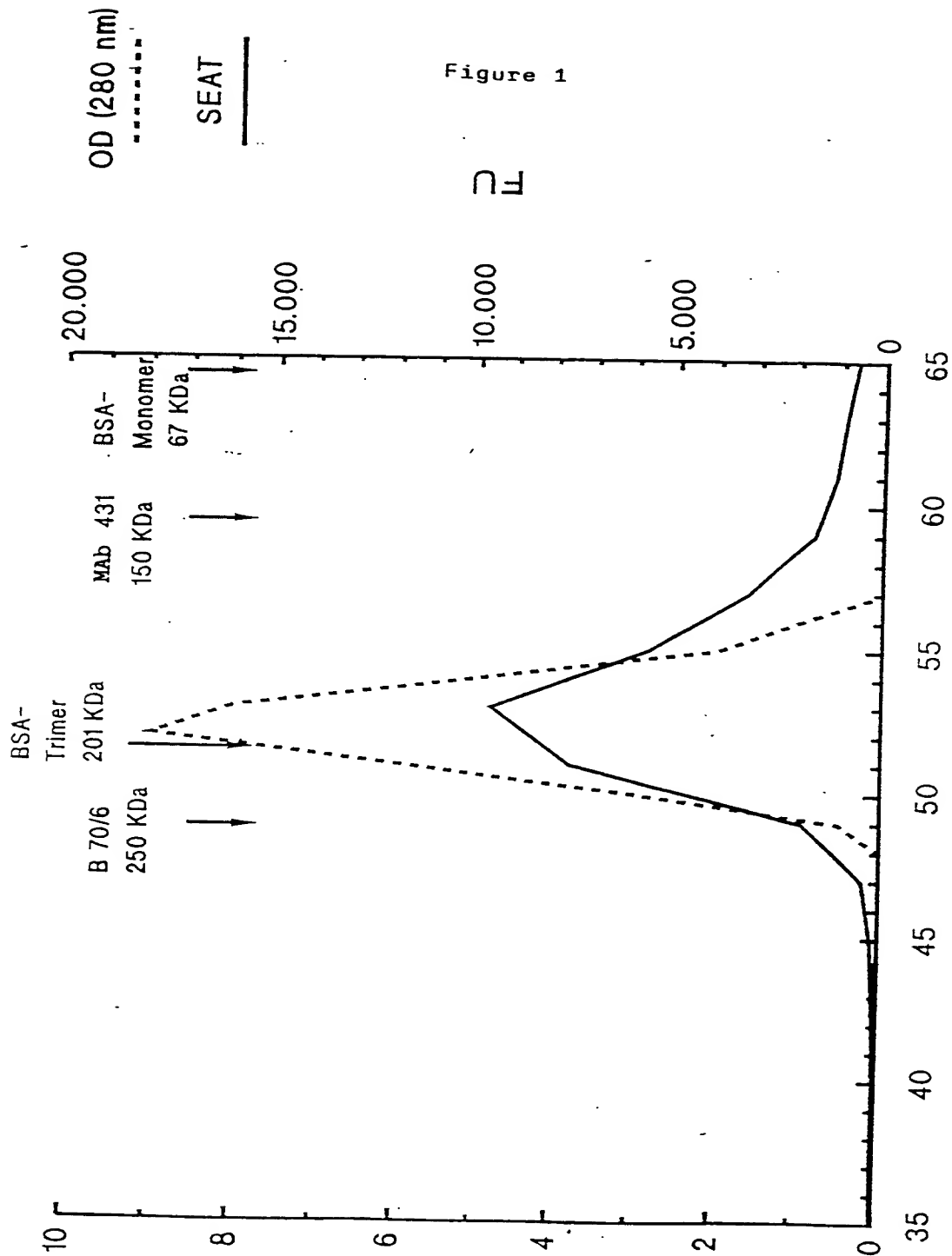


Figure 1

Sample: 200 ng of sFv-huβGluc fusion protein in 25 μl

Figure 2

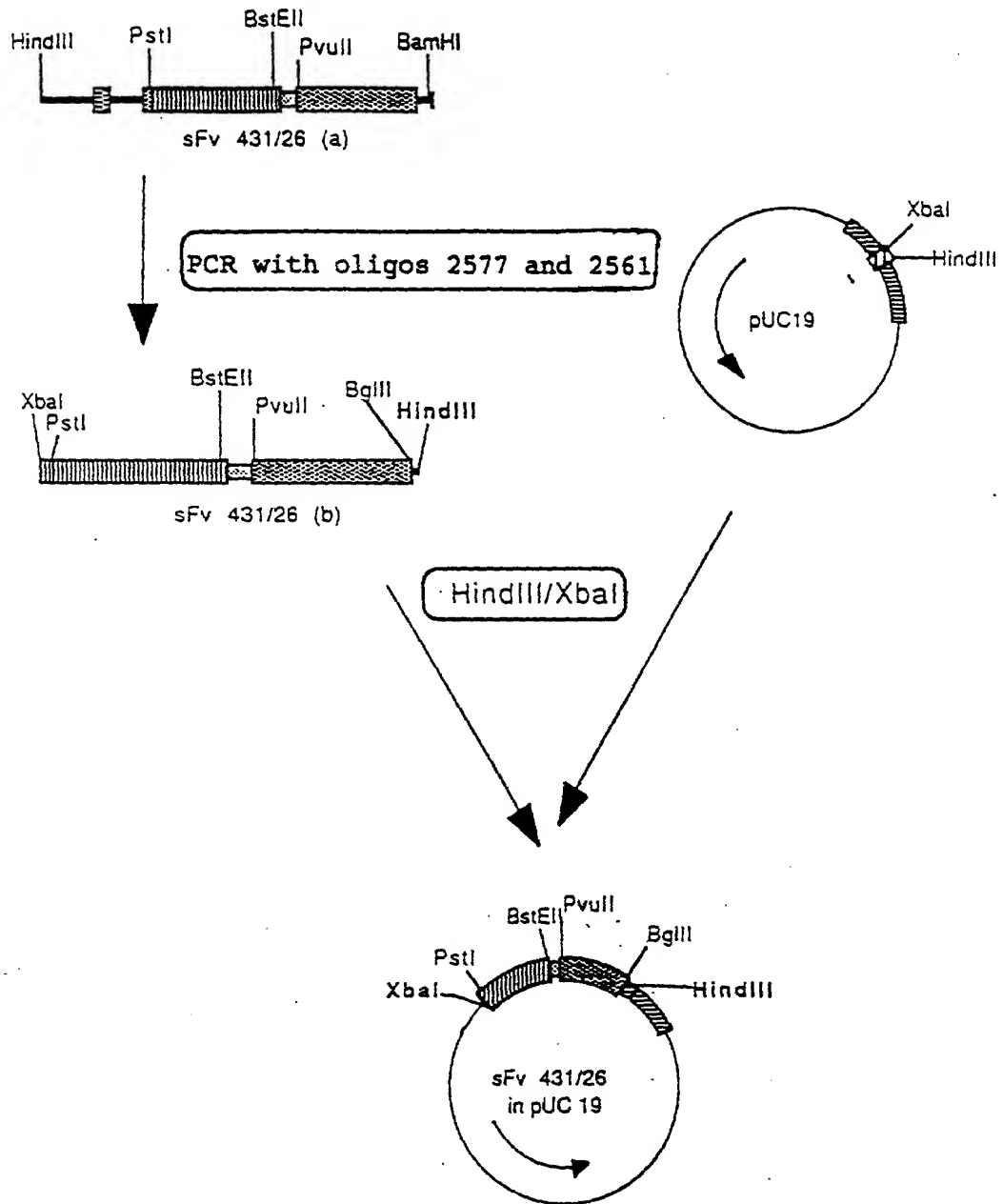


Figure 3

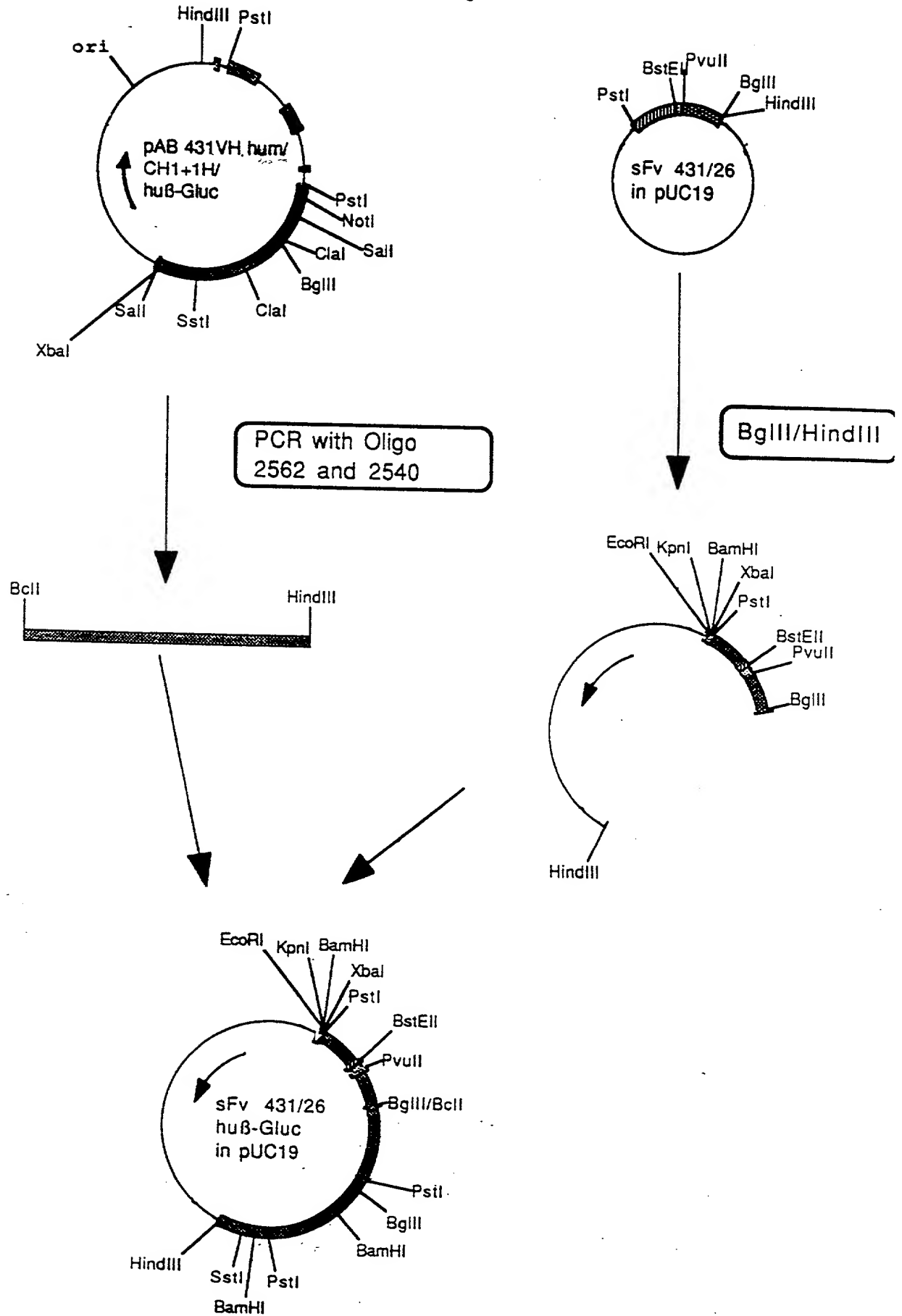


Figure 4

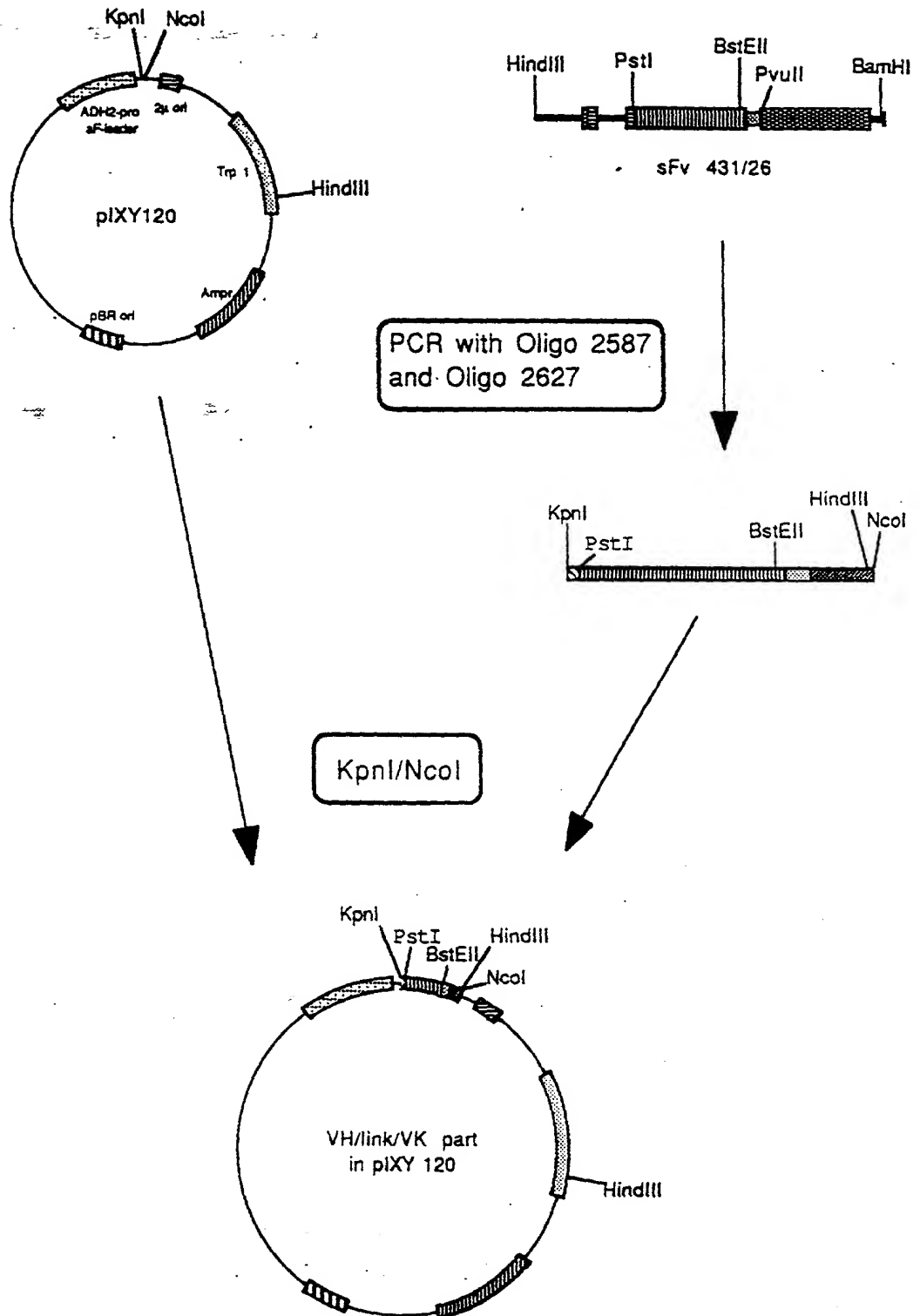


Figure 5

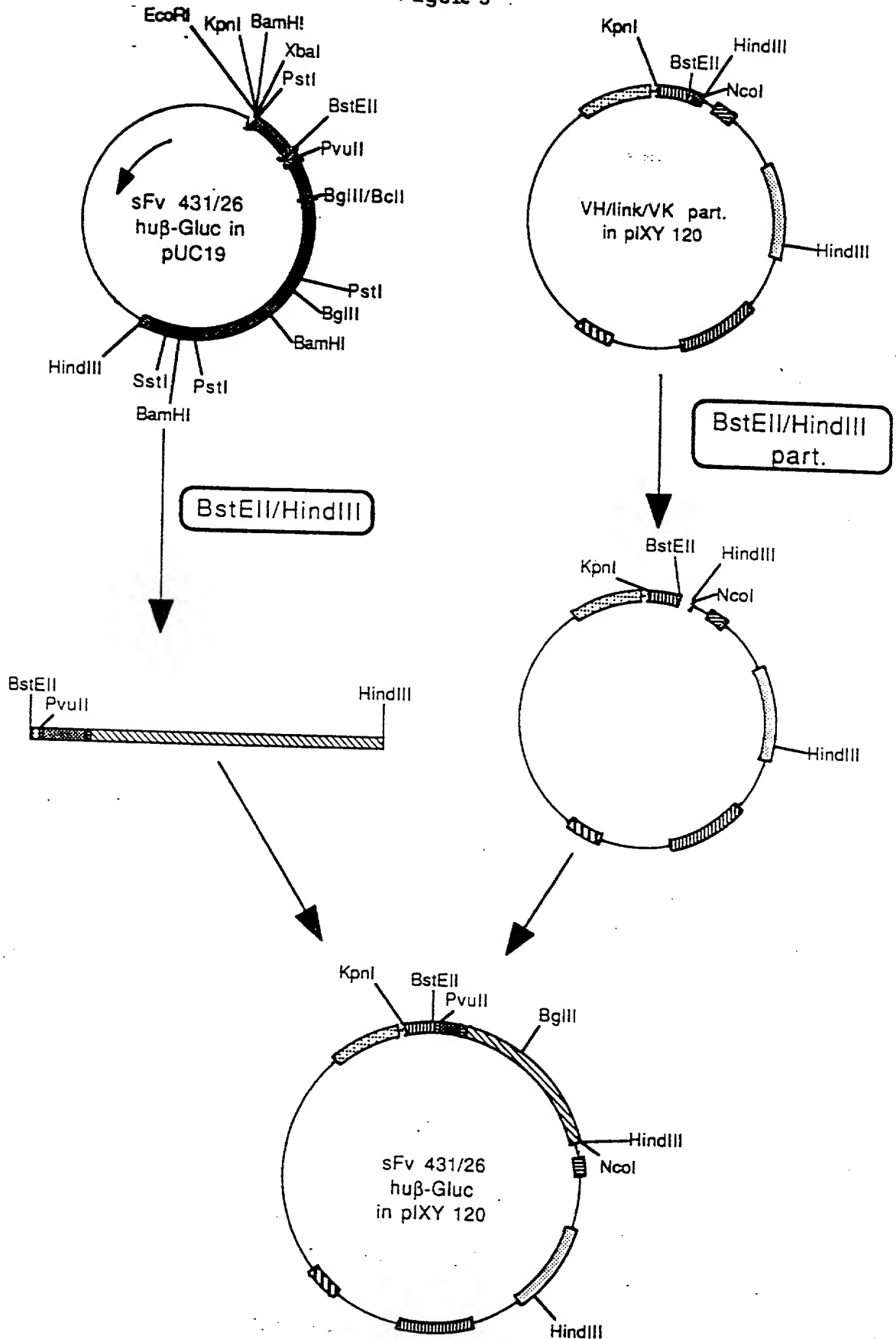


Figure 6

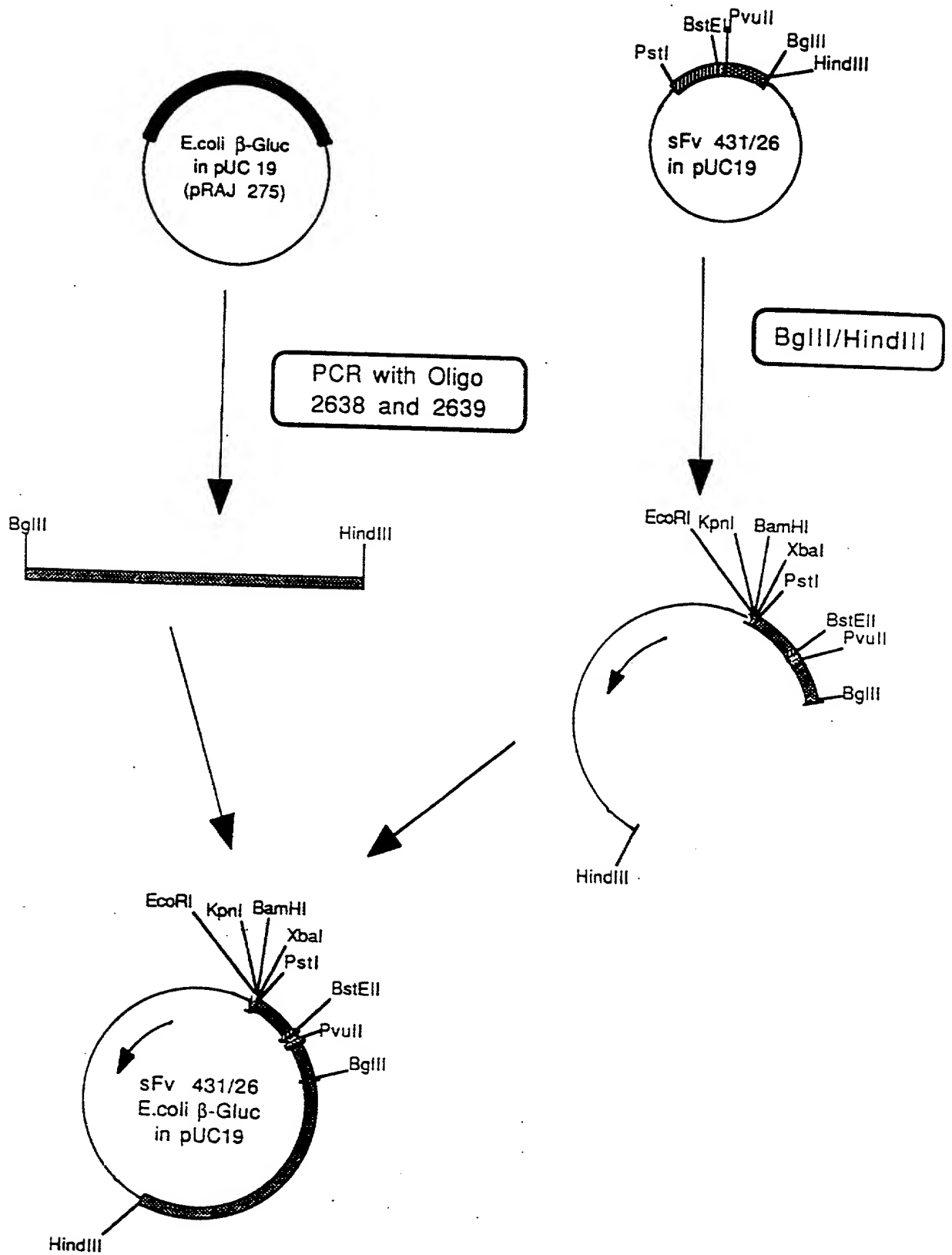


Figure 7

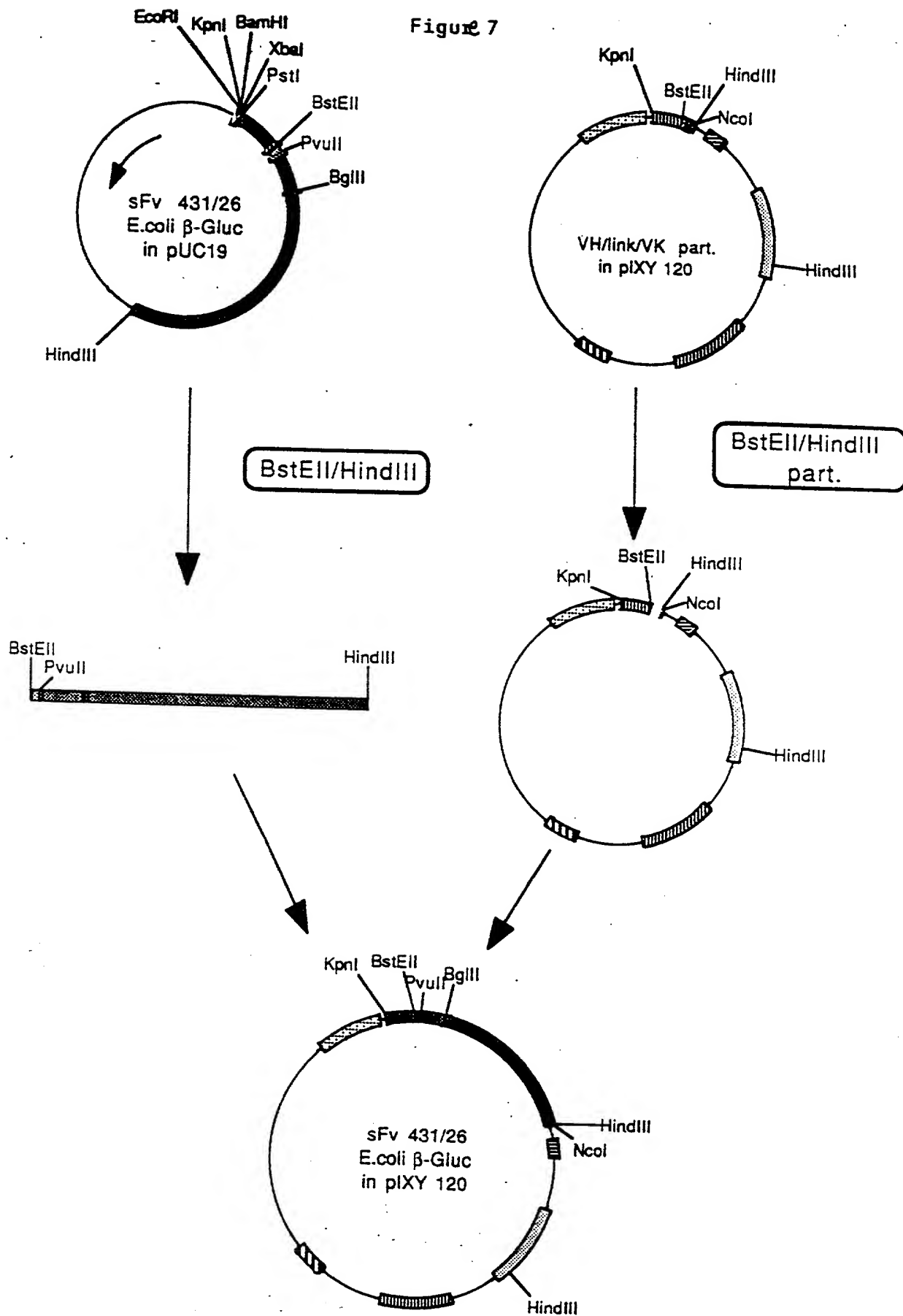


Figure 8

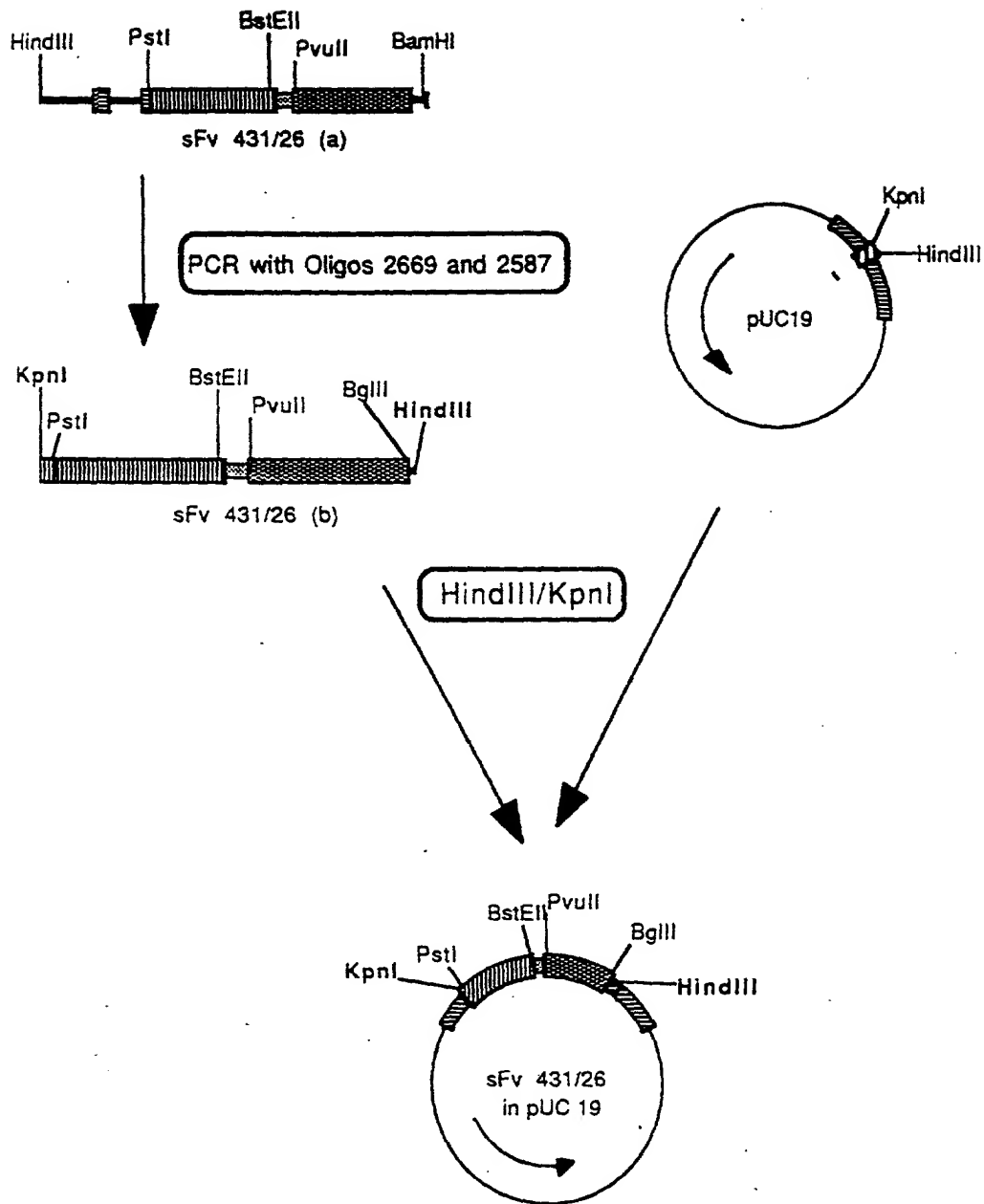


Figure 9

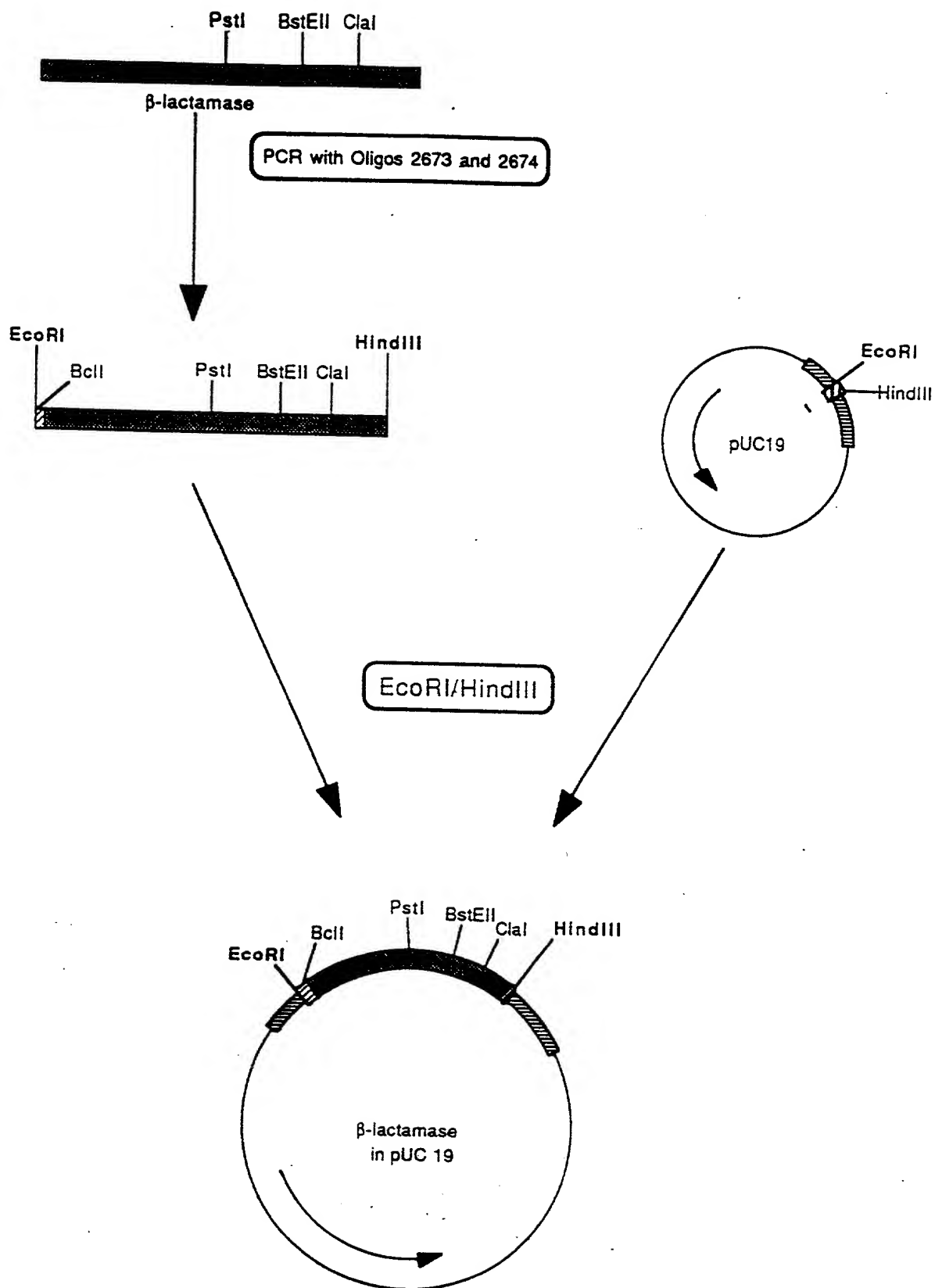


Figure 10

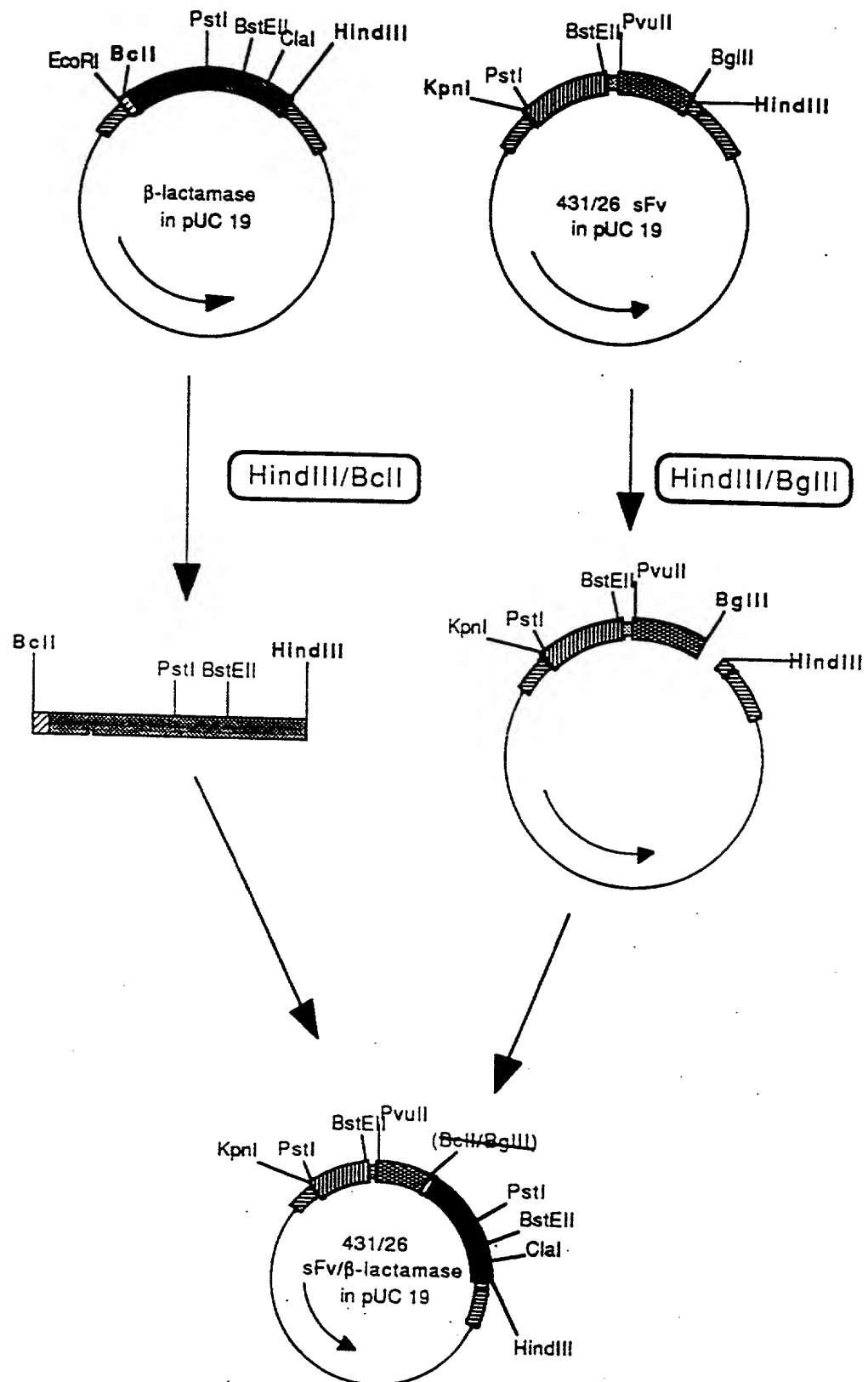
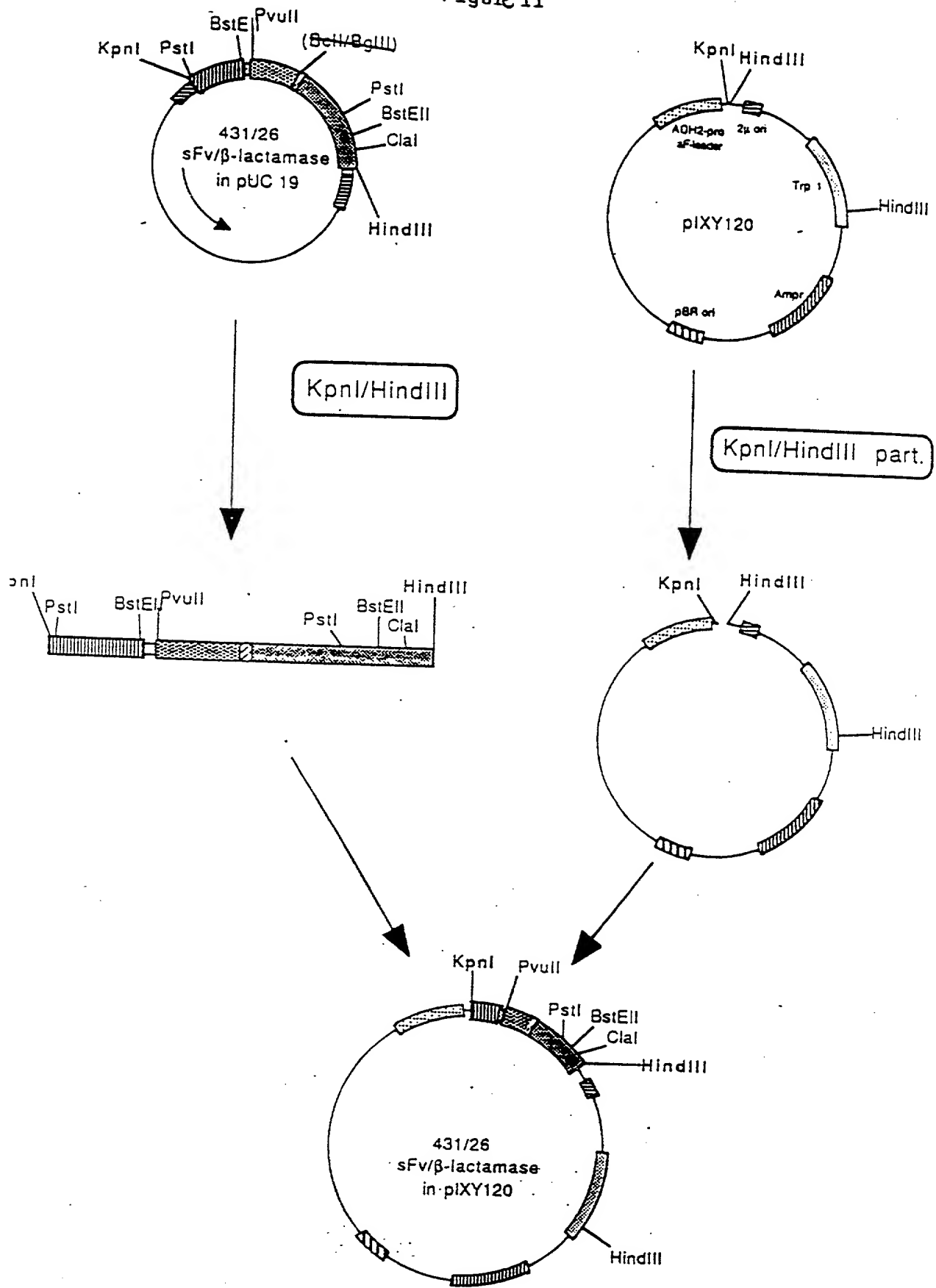


Figure 11



## DECLARATION FOR PATENT APPLICATION

As below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

*Fusion protein for prodrug activation*

(Case Hoe 92/B 024 - Ma 957)

the specification of which is attached hereto / was filed

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims.

I acknowledge the duty to disclose information which is material of the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application in which priority is claimed:

Prior Foreign Application(s) for which Priority is claimed:

Germany P 42 33 152.8 of October 2, 1992

And I hereby appoint

Douglas B. Henderson, Reg. No. 20,291; Arthus S. Garrett, Reg. No. 20,338; Jerry D. Voight, Reg. No. 23,020; Herbert H. Mintz, Reg. No. 26,691; Thomas L. Irving, Reg. No. 28,619; Thomas W. Winland, Reg. No. 27,605; Martin I. Fuchs, Reg. No. 28,805; Susan H. Griffen, Reg. No. 30,907; Richard B. Racine, Reg. No. 30,415; Thomas H. Jenkins, Reg. No. 30,857; Carol P. Einaudi, Reg. No. 32,220; Lawrence M. Lavin, Reg. No. 30,768; Frank E. Caffoe, Reg. No. 18,62 all of the firm of FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, Reg. No. 22,540, my attorneys, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to file continuation and divisional applications thereof, to receive the Patent, and to transact all business in the Patent and Trademark Office and in the Courts in connection therein, and specify that communications about the application are to be directed to the following correspondence address:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signed Marburg, Germany, September 17, 1993

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